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Cytokine expression & TGF-beta signaling in cervical cancer

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Citation
Kloth, J. N. (2009, January 28). *Cytokine expression & TGF-beta signaling in cervical cancer*. Retrieved from https://hdl.handle.net/1887/13438

Version:	Corrected Publisher's Version
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Downloaded from:	https://hdl.handle.net/1887/13438

Note: To cite this publication please use the final published version (if applicable).

Cytokine expression and TGF-β signaling in cervical cancer

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 28 januari 2009
klokke 13.45 uur

door

Judith Nathalie Kloth
geboren te Delft
in 1974

Promotiecommissie

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The research described in this thesis was performed at the Department of Pathology Leiden University Medical Center

The studies described in this thesis were supported by the Dutch Cancer Society (grant no RUL2001-2465).

Financial support of this thesis was provided by:
Dutch Cancer Society
Stichting Nationaal Fonds tegen Kanker
J.E. Jurriaanse Stichting

ISBN: 978-90-7138-279-6
Design cover: CGH array image
Print: Gildeprint Drukkerijen B.V.

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General Introduction

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1 Cervical Cancer: pathogenesis

1.1 Incidence and Epidemiology

Cervical cancer is the second most common cancer amongst women world-wide with an incidence of around 500,000 new cases each year [1]. Developing countries account for 80% of cervical cancer cases [1,2]. The incidence rates vary among geographical areas and depend on factors like early age at sexual initiation, high number of sexual partners, high frequency of exposure to other risk factors (e.g., smoking, other sexually transmitted diseases and poor hygiene) and especially access to routine screening programs [3]. In developed countries, the incidence of cervical cancer is markedly reduced because of the introduction of large-scale cytological screening programs that allow detection and treatment of premalignant disease.

1.2 Classification

Cervical cancer generally originates in the transformation zone, the area of the cervix where the ectocervix and endocervix meet. Carcinomas of the cervix can be classified into squamous carcinomas, adenocarcinomas, adenosquamous carcinomas and neuro-endocrine tumors [4]. About 80% of epithelial cervical tumors are squamous cell carcinomas [5]. The development to cervical cancer is a multi-step process which commences with precursor lesions. The preneoplastic dysplastic changes of squamous carcinoma are referred to as cervical intraepithelial neoplasia (CIN). CIN is divided into three grades, known as CIN1, CIN2 and CIN3. These lesions are classified histologically on the basis of the proportion of epithelial cells with atypia that increases with grade. Most instances of CIN do not progress to invasive carcinoma, probably because human papillomavirus (HPV) infections and CIN are cleared by the immune system [6,7]. Only 1% of cases with CIN1, 5% of cases with CIN2 and 12% of cases with CIN3 will ultimately progress to cancer [8]. Adenocarcinomas represent 10-15% of cervical tumors. These have a glandular origin and their precursor lesion is referred to as cervical glandular intra-epithelial neoplasia (CGIN) and adenocarcinoma *in situ*. Staging of cervical carcinomas is based on clinical evaluation using the 'International Federation of Gynecology and Obstetrics' (FIGO) classification system.

1.3 Carcinogenesis

Human Papillomavirus

Persistent infection with high-risk HPV is the basic cause of cervical cancer [9,10]. More than 150 different HPV genotypes have been defined including 40 anogenital types, at least 13 of these are oncogenic [11,12]. HPV 16 and 18 are the most common HPV types in cervical cancer.

Infection with HPV starts when the virus has gained access to the keratinocytes in

the basal cells of the cervical epithelium. For its life cycle, HPV depends on the differentiation of the keratinocytes. The expression of 3 viral proteins, E5, E6 and E7, occurs as the cervical epithelial cells go through differentiation. These viral early proteins stimulate cell cycle reentry, to guarantee a DNA replicative machinery, on which the virus relies for replication of its genome. E6 and E7 viral proteins interfere with the function of p53 and proteins of the retinoblastoma family. These proteins control cell cycle proliferation and represent important tumor suppressor genes [13]. By deregulating the normal cell cycle, the virus initiates premalignant lesions. The HPV DNA is usually extrachromosomal in benign cervical precursor lesions. The integration of HPV DNA into host-cell DNA of proliferating cells, leading to increased stability of E6 and E7 viral transcripts [14], is likely to be a critical event in cervical carcinogenesis [15].

Genetic factors

Only a small fraction of women with HPV infection in the transformation zone will ultimately develop cervical cancer, indicating that other factors are involved in carcinogenesis. Cervical tumors are characterized by complex genetic alterations throughout the genome. These might involve inactivation of tumor suppressor genes, resulting in loss of function, and amplification or activation of oncogenes, resulting in a gain of function. The most common chromosomal regions with amplification are 1q, 3q, 5p and 8q. Candidate oncogenes in these regions are PI-3 kinase/AKT (3q), c-Myc (8q) and TERT (5p). These genes are involved in cell growth and survival and become activated when amplified [16-18]. Loss of genetic material occurs frequently at chromosomal region 2q, 3p, 4p, 4q, 5q, 6p, 6q, 11q, 13q, and 18q [19]. Possible tumor suppressor genes in these chromosomal regions are FHIT (3p) [20], involved in cell cycle control and apoptosis, NOL7 (6p), possibly involved in inhibition of angiogenesis [21], and human leukocyte antigens (HLA) class I (6p), involved in antigen presentation [22]. Inherited susceptibility to cervical cancer may have some influence. Families in which more than one woman has cervical cancer are rare, although there is evidence that having a sister or a mother with cervical cancer increases a woman's risk of cervical cancer two-fold [23].

Factors associated with the immune system

Variations in the capacity to clear an HPV infection by an effective immune response can be one explanation why certain HPV infections give rise to lesions. The importance of an adequate immune response to prevent the development of cervical cancer is underlined by observations that immunosuppressed women, HIV/AIDS patients [24] and recipients of organ transplants [25,26], show an increased risk of cervical cancer. This implies that a reduced risk of cancer would be expected in women with increased immunologic activity, e.g. allergic individuals. Indeed, for women having sons with allergy (rhinoconjunctivitis, asthma or eczema) the risk of cervical cancer is decreased [27]. Particular alleles involved in immune responses may confer protection or susceptibility to cervical cancer, such as the genes for ERAP1, TAP2 and LMP7,

involved in antigen processing [28]. Also, certain alleles of the polymorphic HLA class I and II genes of the major histocompatibility complex (MHC), involved in antigen presentation, seem to display protection or susceptibility in terms of high-grade CIN development [29,30]. Carriers of commonly reported protective HLA class II alleles displayed lower viral load, short-term HPV infection and a decreased risk of cervical carcinoma *in situ* [30].

2 The immune system

2.1 Immune response

The immune system consists of an innate and acquired branch. Innate immunity is non-specific and functions as a first line of defense. There are different types of defensive barriers within the innate immunity: anatomy (e.g. skin and mucus); physiology (e.g. temperature and pH); humoral factors, such as the complement system and cells (monocytes, neutrophiles and macrophages) that can phagocytose (ingest) particles or microorganisms. The acquired immune system is activated by the innate immune system and is capable of recognizing and remembering specific antigens. Thereby it protects the body against the clinical manifestations of subsequent infections with the same microorganisms. The acquired immune system is represented by a cellular and a humoral section, in both sections lymphocytes play a central role. Humoral immunity is mediated by antibodies, secreted by plasma cells (differentiated B lymphocytes). Recognition of an antigen by a receptor molecule on its surface, leads to activation of B lymphocytes and the generation of plasma cells. By binding antigens of foreign agents (e.g. bacteria), antibodies flag these for destruction [31]. Antibodies can also neutralize toxins or viruses by preventing adhesion of these pathogens to host cells. T lymphocytes, in which T helper cells and cytotoxic T lymphocytes (CTL) are identifiable sub-groups, play a central role in cell-mediated immunity. Recognition of a foreign antigen occurs by a T-cell receptor in association with antigen-presenting proteins, called HLA. The HLA proteins act as “signpost” that display fragmented pieces of an antigen (peptides), either self or nonself, on the cell’s surface. This enables the immune system to distinguish between normal and abnormal cells [32]. Antigen can be presented either by HLA class I or HLA class II proteins. HLA class I is expressed on most cell types whereas HLA class II is expressed predominantly on antigen presenting cells (APC) such as B lymphocytes, dendritic cells and activated macrophages. T helper cells recognize antigen in context of HLA class II proteins. Upon activation, T helper cells release cytokines that influence the activity of other cell types such as B lymphocytes, CTL and phagocytic cells. Therefore, T helper cells play a central role in the regulation of innate and acquired immunity. CTL recognize antigen in context of HLA class I proteins. Recognition occurs by presentation of foreign peptides in the context of HLA class I displayed on cells. Subsequently, CTL kill these cells by releasing cytotoxins that form pores in the cell membrane, inducing activation of an intracellular suicide mechanism, a process

called apoptosis [33]. Thus a cell-mediated immune response is essential for killing of virus-infected cells, intracellular bacteria, fungi and cancer cells.

Besides recognition of antigen, a second signal is usually required for activation of B- and T lymphocytes. In case of T lymphocytes, co-stimulation occurs through engagement of CD28 with B7 molecules on APC [34]. Cytokines, secreted by activated T helper lymphocytes, are co-stimulatory signals necessary for growth and differentiation of B lymphocytes and CTL. In the absence of co-stimulation, anergy, the inability of immune cells to mount an adequate immune response against an antigen, can occur. Different T helper cell subsets, designated Th1, Th2 and Th17 can be distinguished by the cytokines they secrete (Fig. 1). Th1 cytokines include IFNγ that activates CTL, thereby stimulating cell-mediated immunity. In contrast, Th2 cytokines, such as IL-4, IL-5, and IL-10, function as a helper for B lymphocyte activation. A Th2 response is often elevated in allergic disease and parasitic infections [35]. Th17 is a recently discovered T cell subset that is involved in the control of infections against some extracellular bacteria [36,37]. The characteristic cytokine expressed by Th17 cells is IL-17. IL-17 mediates a pro-inflammatory response by inducing the expression of other cytokines (e.g. IL-6) and chemokines (e.g. IL-8) [38]. Furthermore, other subpopulations of T lymphocytes, named suppressor or "regulatory" T lymphocytes can suppress the immune system by producing inhibitory cytokines such as IL-10 and TGF-β. T lymphocytes can regulate the intensity of an immune response by switching from immune-promoting cytokines (e.g. IFNγ) to inhibiting cytokines (IL-10) in a later stage of the response.

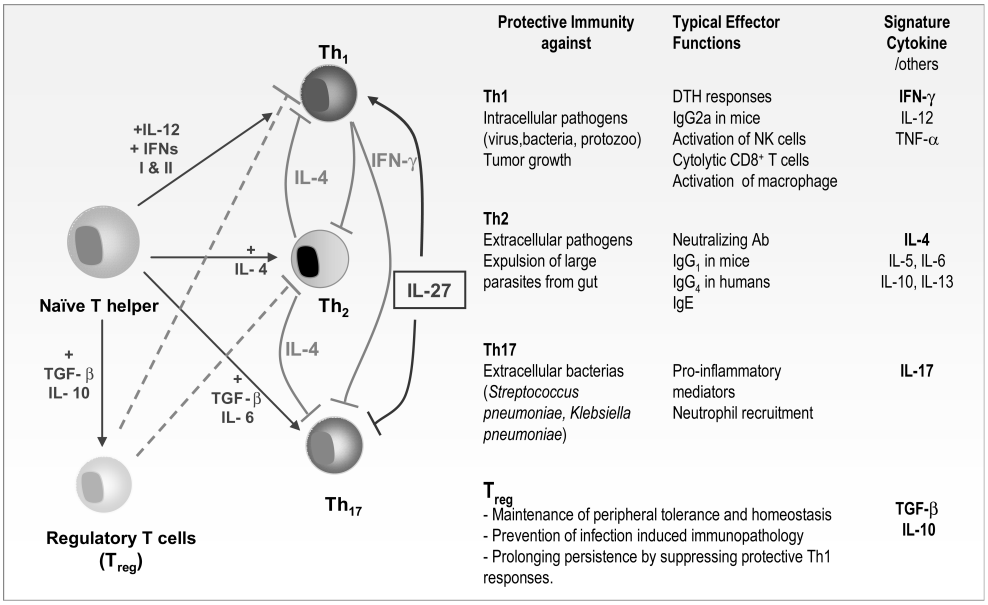


Figure 1. Cytokines involved in differentiation of T helper lymphocyte subsets. Abbreviations: DTH, delayed type hypersensitivity; Ab, antibodies. From Chabalgoity et al., 2007 [35].

2.2 Immune response in cervical cancer

HPVs do not provoke an adequate humoral immune response. Naturally arising antibodies against HPV do not seem to be effective in preventing subsequent infections of homologous or genetically related HPV types [39]. On the contrary, antibodies against HPV capsid proteins that arise after vaccination can be protective at preventing infections of homologous or genetically related HPV types [40,41]. Nevertheless, antibodies do not play an important role in the regression of established HPV infections and cervical lesions [42]. Cellular immune responses however are likely to be an important effector mechanism for the clearance of established infections [43]. HPV-specific T cell immunity is frequently detected in healthy individuals. Presence of T cell immunity therefore plays a role in protecting against persistent HPV infection and hence in the development of cervical malignancies [44]. In patients with cervical lesions and cervical cancer, tumor-specific T lymphocytes against the two HPV encoded oncoproteins, E6 and E7, were detected at low levels in the peripheral blood of approximately 50% of the patients [45-47]. HPV-specific systemic immunity in patients may not be beneficial since it has been associated with a non-inflammatory cytokine profile and did not correlate with prognostic factors [47,48]. In contrast, presence of high numbers of tumor-infiltrating lymphocytes has been associated with a survival advantage in patients with cervical cancer [49,50]. However, recent data suggests that the ratio between infiltrating CTL and infiltrating regulatory T lymphocytes is also an important determinant for prognosis [48],[51]. By suppressing proliferation and cytokine production of CTL, these regulatory T lymphocytes may well interfere with the tumor-specific immune response [52].

3 Immune escape

Due to the presence of foreign proteins of viral origin, and the higher incidence of cervical cancer in immunosuppressed women, cervical cancer is considered an immunogenic cancer. However, HPV have developed a number of immune evasive mechanisms and are therefore poor stimulators of the immune system [53]. HPVs minimize exposure to the immune system by executing viral replication and assembly only in a fully differentiated cell. Consequently, there is no opportunity for APC to engulf virions and present virus-derived peptides to the immune system. Also, there is no blood-borne phase of infection. HPVs do not infect and replicate in APC, nor lyse keratinocytes and therefore do not elicit any pro-inflammatory signals [54]. Furthermore, unlike APC, keratinocytes have low levels of HLA class I and class II molecules, and lack ‘co-stimulator’ molecules such as B7 [55]. The absence of costimulatory and proinflammatory signals is detrimental for the development of an effective immune response against HPV and may result in anergy. Not only do HPV early proteins interfere with cell signaling, targeting genes involved in cell cycle or apoptosis, but also interfere with antigen presentation and expression of cytokines [56]. During the development and progression of cervical cancer also alterations in the cell on (epi)genetic level, influence immune evasion. Two important mechanisms

will be discussed more in detail in the next 2 paragraphs.

3.1 Alterations in HLA class I expression

An important mechanism of immune escape in cervical cancer is HLA class I loss. HLA class I comprises a highly polymorphic heavy chain, encoded by a HLA-A, HLA-B or HLA-C genes located on chromosome region 6p21.3 and a light chain, β2-mircoglobulin. The extracellular part of the heavy chain contains a peptide-binding groove, in which peptides bind in an allele-specific manner. Since HLA molecules are responsible for the presentation of foreign antigens, these molecules play a central role in the immune recognition and subsequent clearance of virus-infected or other deviating cells [57]. Thus loss of HLA class I enables tumor cells to evade recognition and lyses by CTL. Especially in case of tumors with a viral origin such as cervical-cancer, development of a powerful immune response could be hindered since viral antigen is not or insufficiently presented. Defects in HLA class I are caused by multiple mechanisms [58]. Total HLA class I loss can be the result of mutations in the β2-mircoglobulin-gene or defects in the molecules of the HLA class I processing and presentation pathway such as peptide transporter TAP1 or TAP2. Low expression of TAP1 and TAP2 have been observed in cervical cancer cell lines and tumors [59,60]. Partial HLA class I loss can be explained by (structural) defects in the particular HLA proteins which can be caused by locus-specific downregulation, possibly owing to shortcomings at the transcriptional level. Also loss of a single class I allele by mutations or deletions and loss of HLA class I haplotype by loss of heterozygosity (LOH) can be the cause for partial HLA class I loss. LOH can be due to mitotic recombination, deletion or chromosomal nondisjunction [58]. A detailed study of HLA class I aberrations in cervical cancer showed that approximately 90% of the cervical tumors had HLA class I defects [22]. The major mechanism for loss of expression was LOH (50%). Also HPV might interfere with HLA expression as a mechanism to escape from host immune surveillance. HPV16 E5 was shown to impede transport of HLA class I complexes to the cell surface and downregulate surface expression of HLA-A and HLA-B [61]. The importance of HLA class I expression in cervical cancer is emphasized by the findings that reduced expression of HLA class I correlates with worse prognosis and overall survival [62,63].

3.2 Immunosuppressive cytokines

Due to their central role in the immune system, cytokines are involved in a variety of immunological and inflammatory diseases, including cancer. In general, cytokines do not function individually but in concert and have pleiotropic and redundant functions. Th1 cytokines are proinflammatory and boost the immune response, whereas Th2 cytokines, which play a role in humoral immunity, are immune-inhibitory towards cell-mediated immunity. Th1 cytokines are indispensable for the generation of an adequate anti-tumor immune response [64]. However, continuous expression of Th1 cytokines such as TNFα can result in a chronic inflammatory process which might promote cancer [65]. Acute inflammation contributes to the regression of cancer whereas chronic

inflammatory diseases are frequently associated with increased risk of cancer [64,66,67]. During a chronic inflammatory process, besides many cytokines (including growth and angiogenic factors), reactive oxygen and nitrogen species are generated that may cause DNA damage and predispose to neoplasia [67]. In the process of cancer development, cytokines produced by tumor cells, immune cells or stroma, facilitate cancer growth [68]. However in immunogenic cancer types, vaccination with tumor cells in combination with proinflammatory cytokines, including TNFα, IFNγ, IL-2 and GM-CSF, does show therapeutic benefit [69-72]. Evidence exists that this is associated with anti-tumor activity by T lymphocyte- mediated responses [72]. The contribution of proinflammatory cytokines to the initiation and development of cancer or prevention of cancer and anti-tumor activities depends on factors like the type of cancer, the tumor stage and the tumor micro-environment. In cervical cancer, several studies provided evidence that the cause of a failing anti-HPV immune response was a decrease in proinflammatory cytokines such as TNFα [47,73-75]. Also, several studies reported a predominant Th2 cytokine pattern in cervical lesions and cancer [73,76]. Polarization towards a Th2 cytokine profile signifies downregulation of a pro-inflammatory immune response and therefore a decreased susceptibility to the effect of CTL.

Expression of immunosuppressive cytokines like IL-10 and transforming growth factor β (TGF-β) is another strategy used by tumors to escape immunosurveillance [77]. These cytokines inhibit the function of CTL and promote a shift towards Th2 cytokine expression, thereby downregulating anti-tumor immunity [76,78,79]. Tumor-derived IL-10 and TGF-β have been shown to induce regulatory T lymphocytes and skew the immune response to Th2 polarity in cervical cancer [76]. It is conceivable that cervical cancers circumvent the development of a potent HPV-specific anti-tumor immune response by not providing the appropriate proinflammatory environment and instead stimulate the expression of immunosuppressive cytokines as TGF-β and IL-10. TGF-β will be discussed in more detail in the next chapter, since a substantial part of this thesis concerns research into this cytokine.

4 TGF-β

TGF-β is a pleiotropic cytokine that apart from its immunosuppressive function, is involved in the regulation of many biological processes including cell growth, development, apoptosis, differentiation, migration, angiogenesis and extracellular matrix production. Three isoforms of TGF-β exist (TGF-β1, TGF-β2 and TGF-β3), which belong to the TGF-β superfamily that among others include activins and bone morphogenetic proteins. In a large number of human cancers, changes in TGF-β signaling are frequently observed [80]. TGF-β signaling can have both positive and negative effects in cancer depending on the cell type, stage of carcinogenesis and the responsiveness of the tumor cell.

4.1 Signal transduction

TGF-β1 is secreted as part of a latent complex into the extracellular matrix [81]. "TGF-β activators" such as thrombospondin, matrix metalloproteinases, plasmin and certain integrins release TGF-β1 from its latent state [78,82]. Once activated, TGF-β family members initiate signaling by interacting with receptor serine/threonine kinases, type I and type II (Fig. 2). Upon ligand binding, the TGF-β receptor type II (TβRII) phosphorylates the TGF-β receptor type I (TβRI) which leads to the activation of its kinase domain and subsequent regulation of the mothers against decapentaplegic drosophila homolog (Smad) proteins. Activated TβRI phosphorylates Smad2 and Smad3 proteins, which form hetero-oligomeric complexes with Smad4. These complexes translocate to the nucleus and, depending on the cell type and their

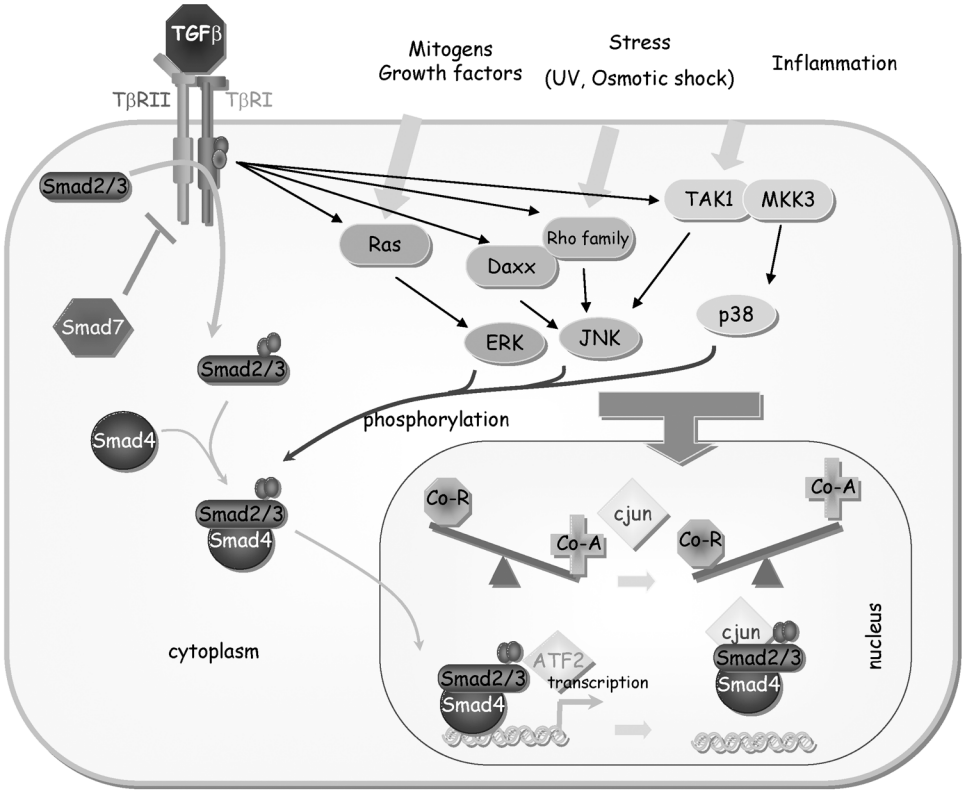


Figure 2. TGF-β1 induced activation of Smad and MAPK and their interactions. Activation of Smad signaling occurs once TGF-β1 binds to the TGF-β receptor type II (TβRII). Subsequently, TβRII phosphorylates TGF-β receptor type I (TβRI) which in turn phosphorylates Smad2/3. Then, activated Smad2/Smad3 bind Smad4 and translocate to the nucleus where the complexes function as transcription factors. Transcription is controlled by the presence of coactivators (Co-A) or corepressors (Co-R). Activation of the 3 MAPKs (ERK, JNK and p38) by TGF-β1 or other stimuli such as growth factors or proinflammatory cytokines, can regulate Smad activation by direct phosphorylation or through downstream effector molecules. Examples of downstream effector molecules are cjun and ATF2 which modulate transcriptional activity. From Javelaud et al., 2005 [84].

interactions with certain transcription factors, activate or repress transcription via direct DNA binding [83].

Evidence exists for a strong integration of Smad signaling within a complex network with other signaling pathways that largely contribute to modifying the initial Smad signals and allows diverse responses to TGF-β1 [84,85]. Smad signaling may even be dispensable for some of the responses initiated by TGF-β1 [86,87]. TGF-β1 has been shown in various cell types to activate extracellular signal-regulated kinase (ERK), p38 and jun kinases (JNK) which are mitogen activated protein kinases (MAPK) [88,89]. ERK-mediated pathways are involved in proliferation and usually anti-apoptotic. In contrast, JNK and p38-signaling, activated by stress stimuli, often induce apoptosis. Activation of MAPK by TGF-β1 can occur directly by TGF-β activated kinase-1 (TAK1) or indirectly, possibly resulting from Smad-dependent transcriptional responses [90]. MAPK can positively and negatively influence TGF-β1 signaling [84]. MAPK negatively influences TGF-β1 signaling by stimulating expression of Smad7, an inhibitor of Smad signaling [91]. Furthermore, phosphorylation of the linker region of R-Smads may block nuclear translocation and signaling by Smads [92]. MAPK are positively implicated in TGF-β1 signaling by interaction of AP-1 transcription factors, downstream components of the MAPK pathway, with Smad complexes to regulate transcription [93]. Also other signaling pathways, including the PP2A/p70S6, Rho GTPases, PI-3 kinase/AKT and Wnt pathways, can either be induced by TGF-β1, or can modulate the outcome of TGF-β-induced Smad signaling [94-97].

4.2 Role in cancer

TGF-β1 as a tumor suppressor

During tumor initiation and early progression TGF-β1 is thought to be a tumor suppressor, whereas late in tumor progression TGF-β1 signaling promotes tumorigenesis (Fig. 3). The anti-tumor response by TGF-β1 early in tumorigenesis is mediated by its growth inhibiting and pro-apoptotic properties via both Smad and non-Smad signaling pathways [85]. TGF-β1 stimulation of epithelial cells has been shown to induce a G1 cell cycle arrest by activating various anti-proliferative responses such as the transcriptional upregulation of cyclin-dependent kinase (CDK) inhibitors p15 and p21 [98,99]. Also, TGF-β1 transcriptionally represses cdc25A [100], a phosphatase that removes inhibitory phosphatidyl groups from CDK, the progrowth transcription factor c-Myc and the inhibitors of differentiation Id1, Id2 and Id3 [101,102]. Pro-apoptotic responses by TGF-β1, often mediated by Smad proteins, are caspase-8 activation via upregulation of the FAS receptor, transcriptional induction of death associated protein kinase and upregulation of the pro-apoptotic proteins Bim and Bmf [103-105]. In view of the above, somatic mutations and loss of expression of various components of the TGF-β-Smad signaling pathway, could lead to resistance of TGF-β1 mediated growth inhibition in cells. In pancreatic cancer, Smad4 is genetically inactivated in approximately 50% of tumors [106]. Also in certain colorectal cancers, Smad4 mutations are often found [107]. TβRII mutations are frequently found in cancers associated with microsatellite instability, such as hereditary non-polyposis

colorectal cancer [108]. Mutations in TβRI and Smad2 occur at low frequency, whereas Smad3 mutations have not been detected so far [80]. Loss of expression of these components was often found to be associated with worse prognosis [109,110]. However, some alterations of the TGF-β1 pathway do not lead to a complete loss of signaling and could result in loss of tumor suppressive effects while retaining the tumor promoting effects [80]. An alternative way of inactivating the tumor suppressor function of TGF-β1 is by overexpressing or amplification of its inhibitors. For example, Smad7 amplification in colorectal cancer and high expression of SMURF2 in esophageal squamous cell carcinoma were found to be poor prognostic markers [111,112]. The proto-oncogene product Ski was shown to promote tumor growth in pancreatic cancer by abrogation of TGF-β1 signaling [113]. Furthermore, oncoproteins c-Myc and Ras were shown to antagonize the growth-inhibitory response of TGF-β1 signaling in epithelial cells [92,114-116].

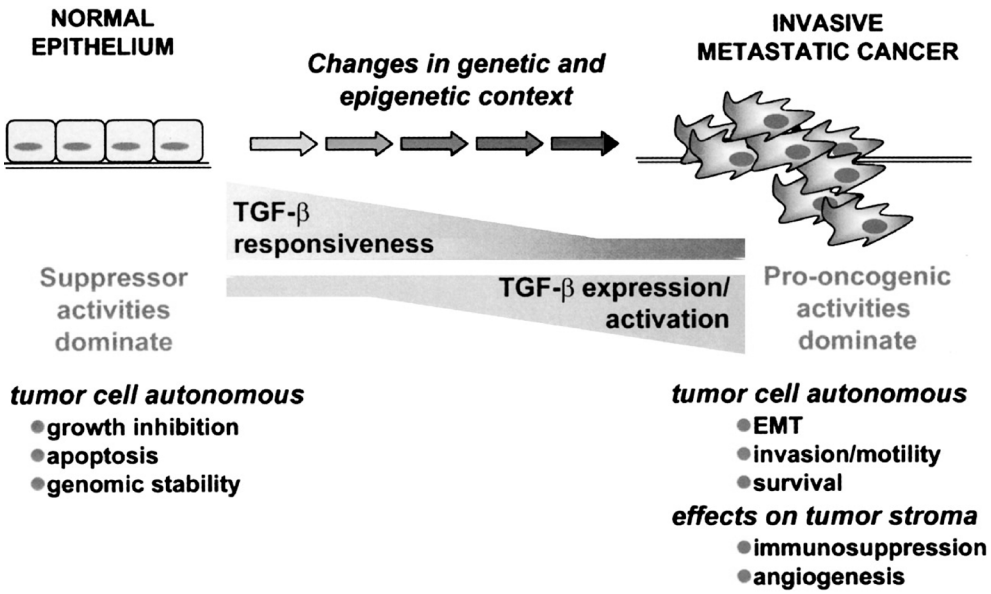


Figure 3. TGF-β1 switches from tumor suppressor in normal and premalignant stages of tumorigenesis to proto-oncogene at later stages of disease. Progression to metastatic disease is usually accompanied by decreased or altered TGF-β1 responsiveness and increased expression/ activation of TGF-β1. Besides changes in the responsiveness to TGF-β1 in tumor cells, TGF-β1 effects tumor stroma in such a manner that tumor growth and metastasis is facilitated. From Roberts et al., 2003 [117].

TGF-β1 as a tumor promoter

During the process of carcinogenesis, cells often lose the anti-proliferative or apoptotic response to TGF-β1 and show increased production of this cytokine. The tumor-promoting role of TGF-β1 is mediated through its effect on tumor cell invasion, angiogenesis, changes in the tumor microenvironment and the cells of the immune system. TGF-β1 can induce an invasive phenotype in cancer cells, characterized by

an epithelial-to-mesenchymal transition (EMT) [118,119]. Cells undergoing EMT exhibit changes in morphology, loose cell-cell and cell-matrix adhesive properties accompanied by degradation of the surrounding extracellular matrix (ECM). A number of studies have shown that the Smad pathway as well as other pathways in TGF-β1 signaling such as ERK and PI-3 kinase/AKT signaling are required for EMT [120-123]. Increased production and activation of TGF-β1 by tumor cells stimulates synthesis of ECM proteins and chemokine production as well as activation of fibroblasts [124,125]. By providing growth factors, ECM proteins and matrix metalloproteinases, fibroblasts support tumor progression [126,127]. Furthermore, TGF-β1 stimulates tumor angiogenesis. Angiogenesis is crucial for tumor growth and invasion, as blood vessels deliver nutrients and oxygen to the tumor cells and allow migration of tumor cells via the blood system, resulting in metastasis. TGF-β1 induces expression of vascular endothelial growth factor (VEGF) [128], thereby stimulating proliferation and migration of endothelial cells [129] and capillary formation of endothelial cells [130]. In addition, TGF-β1 possesses chemoattractant activity for monocytes which release angiogenic cytokines. Escape from immunosurveillance is mediated by inhibition of T lymphocyte proliferation through expression of cell cycle regulators and blockade of the production of IL-2, a cytokine known to activate T lymphocytes, NK cells and other types of the immune system [131]. TGF-β1 also controls T- and NK-cell effector functions by attenuating the cytolytic activity of these cells, inhibiting expression of IFN-γ, necessary for the stimulation of a Th1 response, and inhibiting the expression of perforin [132-134]. Furthermore, antigen presentation by differentiating dendritic cells, which are powerful APC, is hampered by inhibiting the expression of HLA class II and costimulatory molecules [135].

4.3 TGF-β1 in cervical cancer

During cervical carcinoma development, a progressive loss of sensitivity to TGF-β1-mediated growth inhibition was found [136]. This seems to be accompanied by increased expression levels of TGF-β1 during the progression from benign to malignant lesions [137-139]. Whereas TGF-β1 did not hinder proliferation of cervical cancer cells [138], an inverse relationship was found between its expression and the amount of tumor infiltrate [140]. Furthermore, TGF-β1 mRNA expression correlated with the amount of intratumoral stroma [140]. Consequently, the tumorsuppressive characteristics of TGF-β1, such as growth inhibition, seem to be lost, whereas the tumor-promoting features of TGF-β1, such as immunosuppression and ECM production, appear to be fully functional in cervical cancer. Mutations in signaling components of the TGF-β1-Smad pathway have been observed in cervical cancer and may play a role in carcinogenesis [141-143]. So far, mutations in Smad4 and TβRII were mainly found in cell lines [141,143,144]. A possible relation between mutation of Smad4, accompanied with loss of protein expression, and resistance to TGF-β1-induced growth inhibition, remains to be elucidated since data are conflicting [141,144]. Interference with TGF-β1 signaling can also occur through HPV E7 oncoproteins which bind Smad proteins and inhibit Smad-induced transcription [145,146]. HPV16 E7 may also interfere with TGF-β1 signaling by blocking TGF-β1

suppression of c-myc transcription via pRb. This has been observed in human foreskin keratinocytes transformed with HPV16 and was associated with resistance to the growth inhibitory effects of TGF-β1 [101]. However, TGF-β1 itself has been shown to inhibit expression of HPV16 E6 and E7, accompanied with cessation of cell proliferation in immortalized genital epithelial cell lines [147]. After malignant transformation, cells became partially resistant to the inhibitory effects of TGF-β1 on cell growth and gene expression of HPV16 E6 and E7 [147]. Thus far, it is unknown whether resistance to TGF-β1-induced growth inhibition is due to mutations in the Smad signaling pathway, interference by HPV oncoproteins or changes in signal transduction due to crosstalk with other pathways. Further insights into the signaling pathways downstream of TGF-β1, and the contributions of these pathways to the specific cellular and context-dependent effects of TGF-β1, may lead to more specific targeting of this pathway in the treatment of cancer.

5 Outline of the thesis

Cervical cancer is a virus-induced tumor that has developed several escape mechanisms to avoid eradication by the immune system. In this thesis we have investigated several of the immune escape routes that cervical cancer cells have acquired. A common feature of cervical cancer cells, occurring in ± 90% of tumors, is their ability to prevent recognition by the immune system through the loss of HLA class I protein. We hypothesized that tumors without HLA class I loss have developed other ways to prevent elimination by the immune system. In these latter cases, expression of immunosuppressive cytokines, such as TGF-β1 or IL-10, could be a mechanism to escape immune surveillance. In **chapter 2**, tumors positive and negative for HLA class I were compared, based on gene expression profile, to investigate escape routes in tumors with HLA class I expression. Microarray was used as a platform to study genomewide gene expression changes between tumors positive and negative for HLA class I. Subsequently, real-time PCR and immunohistochemistry were used to confirm expression changes. An alternative mechanism for immune escape in cervical cancer, besides loss of HLA class I and expression of immunosuppressive cytokines, is loss of proinflammatory cytokine expression. The presence of the proinflammatory cytokine TNFα may also be detrimental for the development of an adequate immune response against HPV.

Therefore, in **chapter 3**, loss of TNFα gene expression, as a mechanism to escape immune clearance, was investigated. The (epi)-genetic characteristics associated with the loss of TNFα expression were studied to investigate if TNFα could be a candidate tumor suppressor gene.

TGF-β1, a cytokine with potent immuno-inhibitory qualities, is excessively produced by cervical cancer cells. As discussed earlier, TGF-β1 suppresses the immune response by several mechanisms. However TGF-β1 also suppresses cell growth, therefore cervical cancer cells need to first acquire insensitivity to TGF-β1 induced cell growth inhibition, thus providing the tumor cells a growth advantage. In **chapter 4,** the gene expression profile (using microarray) of cervical cancer cell lines with a

different sensitivity towards TGF-β1 was investigated. To investigate if TGF-β1 insensitivity in cervical cancer can be due to alterations in the canonical Smad pathway, loss of Smad2, Smad2-P and Smad4 protein expression was investigated by immunohistochemistry in a large cohort of clinical samples as described in **chapter 5**. Tumors with low expression of Smad2-P and Smad4 were investigated for genetic inactivation, loss of heterozygosity and in case of Smad4 for promoter methylation to determine if these genes are candidate tumor suppressor genes in cervical carcinogenesis.

Cervical tumors are characterized by complex genetic alterations involving deletions and amplifications that can affect many chromosomal regions. Also genes involved in immune responses can be affected, as has been shown for HLA class I genes. Currently, large-scale DNA copy number, genotyping and gene expression profiling methods are available to study the genome. To obtain an overview of genome-wide molecular alterations in cervical cancer and to uncover genes involved in cervical carcinogenesis, including genes related to the immune response, we investigated DNA copy number and genotype alterations in cervical cancer cell lines in detail using array comparative genomic hybridization (CGH) and single nucleotide polymorphisms (SNP) array, as described in **chapter 6**. Furthermore, an effect of copy number alteration on gene expression was investigated. Finally, in **chapter 7** the results of the above mentioned studies are discussed and summarized.

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2

Elevated expression of SerpinA1 and SerpinA3 in HLA positive cervical carcinoma

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Journal of Pathology. 2008; 215(3): 222-230

In cervical cancer, an important mechanism by which tumor cells escape immune surveillance is loss of HLA class I, enabling tumors to evade recognition and lysis by cytotoxic T lymphocytes. Some tumors, however, escape from immune surveillance without accumulating defects in antigen presentation. We hypothesized that tumors with no or partial loss of HLA class I develop alternative mechanisms to prevent immune surveillance. To investigate this hypothesis, genome-wide expression profiling using Illumina arrays was performed on cervical squamous cell carcinomas showing overall loss of HLA class I, partial and normal HLA class I protein expression. Statistical analyses revealed no significant differences in gene expression between tumors with partial (n = 11) and normal HLA class I expression (n = 10). Comparison of tumors with normal/partial HLA class I expression (n = 21) with those with overall loss of HLA class I expression (n = 11) identified 150 differentially expressed genes. Most of these genes were involved in the defense response (n = 27), and, in particular, inflammatory and acute phase responses. Especially SerpinA1 and SerpinA3 were found to be upregulated in HLA positive tumors (3.6 and 8.2 fold, respectively), and this was confirmed by real-time PCR and immunohistochemistry. In a group of 117 tumors, high SerpinA1 and SerpinA3 expression in association with normal/partial HLA expression correlated significantly with poor overall survival ($p = 0.035$ and $p = 0.05$, respectively). This study shows that HLA positive tumors are characterized by a higher expression of genes associated with an inflammatory profile and that expression of the acute phase proteins SerpinA1 and SerpinA3 in HLA positive tumors is associated with poor survival.

Introduction

The development of cervical cancer is a step-wise process, preceded by dysplastic lesions known as cervical intraepithelial neoplasia (CIN) stage I, II and III. Infection with high-risk human papilloma virus (HPV) plays an essential role in the development of cervical carcinoma [1]. In addition to persistent HPV infection, other factors are necessary and contribute towards the progression of CIN lesions to invasive cancer. The host immune response in particular plays a critical role in determining the progression or regression of HPV-associated CIN lesions [2,3].

Upon infection with HPV, the host initiates a virus-specific cell-mediated immune response, which in most instances leads to regression of the CIN lesions [4]. Cytokines play an important role in regulating the immune response towards either anti-tumor immunity or immune suppression [5]. In addition to the involvement of certain immunostimulatory cytokines, the development of an adequate cell-mediated immune response requires the proper presentation of HPV-associated proteins by human leukocyte antigens (HLA) class I proteins on the cell surface of infected keratinocytes.

Many tumor types have developed mechanisms to escape immune surveillance that involve abnormal HLA class I antigen expression; this may range from loss of a single HLA class I allele to complete loss of HLA class I antigen expression [6]. Loss of HLA class I expression occurs in the majority of cervical cancers via several underlying mechanisms, of which loss of heterozygosity in the HLA region is the most frequent event (50%) [7].

Cervical cancer cells that do express HPV peptides in HLA class I molecules may maintain survival by creating an anergic environment in which the recruited cells from the immune system are incapable of eradicating the tumor cells. Expression of immunosuppressive cytokines, such as TGF-β and IL-10 by tumor cells or suppressor T cells, has been shown to inhibit the generation and function of cytotoxic T cells [8].

Thus far, immune escape mechanisms in cervical carcinoma have been studied primarily individually. Therefore it is unknown whether different immune escape mechanisms occur simultaneously or arise independently. We hypothesize that tumors that retain HLA class I expression employ alternative defense mechanisms to prevent eradication by the immune system.

Materials and methods

Tissue samples

Formalin-fixed, paraffin-embedded squamous cervical carcinomas from 68 patients who underwent radical hysterectomy with bilateral lymphadenectomy between 1991 and 2005 at Leiden University Medical Center, were collected. From these 68 patients, 32 snap-frozen tissues were chosen for subsequent gene expression profiling. The percentage of tumor cells was estimated by visual examination of HE-stained frozen tissue sections (5 μm). Only tissue blocks with areas that contained at least 60% viable tumor cells were selected. The inflammatory infiltrate was microscopically

assessed on HE slides and scored as mild, moderate or extensive. A separate group of 117 formalin-fixed, paraffin-embedded cervical carcinomas embedded in a tissuearray in triplicate, obtained between 1985 and 1999, was used to associate immunohistochemical data with clinicopathological parameters. There was no overlap between the tumors on the tissue array and the tumors used for gene expression profiling. HPV DNA detection was performed by the INNO-LIPA prototype research genotyping assay (Innogenetics, Gent, Belgium) as described previously [9]. Correlation of expression data with clinicopathological parameters or survival was performed with χ^2 and Kaplan Meier tests. All tissues were retrieved from the archives of the Department of Pathology, Leiden University Medical Center. None of the patients had received radio- or chemotherapy prior to surgery. The local Institutional Review Board approved the use of human tissue material for research.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections as described [10]. For HLA class I staining, antibodies against HLA-A (HCA2); HLA-B and HLA-C heavy chains (HC10, both antibodies were a kind gift from Prof. J. Neefjes, Dutch Cancer Institute); and β2m (A 072; DAKO, Copenhagen, Denmark) were used. Normal epithelium, stroma and infiltrating leukocytes served as an internal positive control. Polyclonal antibodies against SerpinA1 (1:12800, A0012, DakoCytomation, Glostrup, Denmark), with proteinase K as antigen retrieval, and SerpinA3 (1:20000, A0022, DakoCytomation) were used for validation of expression microarray data. Immunohistochemical staining was scored based on intensity and percentage of positive tumor cells [11]. The intensity of each marker was scored as 0, 1, 2, or 3, indicating absent, weak, clear or strong expression, respectively. The percentage of positive cells was scored on a scale of 0 (0%), 1 (1-5%), 2 (5-25%), 3 (25-50%), 4 (50-75%), and 5 (75-100%). Samples were categorized in one of three categories of expression based on the sum of the scores: strong expression (total score 6-8), partial loss (3-5) and total loss (0-2). Fisher's exact tests were computed to determine differences between groups.

Microsatellite analysis

Genomic DNA was isolated from flow-sorted tumor cell subpopulations derived from cervical carcinomas as previously described [12]. Eight fluorescein-labeled primer pairs for microsatellite markers, including MOGC, D6S265, C125, TY2A, TNF, D6S273, D6S294 and D6S1666 (http://www.GDB.org/) were used. PCR, electrophoresis and analysis was performed as described previously [13]. In comparing normal and tumor DNA, a reduction of more than 50% of one allele was assigned as LOH. Tumors were considered to have LOH if all or 2 of the 3 markers spanning the HLA class I region (MOGC, D6S265 and C125) harboured LOH.

RNA extraction, labelling and microarray hybridization

Total RNA was isolated from frozen tissue slides with Trizol (Life Technologies, Carlsbad, CA) and purified as described [14]. The integrity and quantity of the total RNA and the labeled cRNA was assessed by spectrophotometric analysis on the

Agilent 2100 Bioanalyzer conductor with the RNA 6000 Nano Assay Kit (Agilent Technologies, Palo Alto, CA). cDNA was synthesized and transcribed into cRNA using the Illumina Totalprep RNA amplification kit following the manufacturer's instructions (Ambion, Austin, Texas). Labeled cRNA was hybridized to Illumina Sentrix-human 6 expression bead-chips. These chips have genome-wide coverage with around 47,000 50-mer probes, at approximately 30-fold.

Microarray analysis

Gene expression levels were quantified using beadstudio gene expression module 2.1, and data were subsequently normalized using the VSN method [15]. Access to complete microarray datasets are available at GEO, accession number GSE10372. Only the well-annotated genes were selected for further analysis (n = 26091). Differential gene expression analysis was performed first of all by a nested F-analysis using limma which is comparable to ANOVA [16]. After combining group B and C (see Results) T-tests in limma were performed. An FDR threshold of 0.05 was used, according to Benjamini and Hochberg [17], to select differentially expressed genes. The Global test was used [18] to determine differences between the tumor groups in certain Gene Ontology Biological processes. The influence of a different degree of inflammatory infiltrate between tumors on gene expression was tested as confounding factor in the Global test.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described previously [14]. Gene expression was normalized using the Genorm program [19]. Primers for the internal control genes *EEF1A1*, *RPL11* and *RPL13* were previously reported [14]. Commercially available primers were used for *SerpinA1*, *SerpinA3* and *CCL4* amplification (Superarray, Frederick, USA). Independent T-tests were performed to determine significant differences in expression between the groups. Spearman's nonparametric correlation coefficients were calculated for the relationship between microarray and real-time expression levels.

Immunofluorescence

Deparaffinized tissue sections were incubated with a mix of HC10 (mouse IgG2a) and SerpinA3 (rabbit polyclonal) antibodies. Proteins were visualized using a combination of fluorescent antibody conjugates (goat-anti-rabbit-IgG-Alexa-546 and goat-anti-mouse-IgG2a-Alexa-488). The images were captured with a confocal laser scanning microscope (Zeiss LSM510; Zeiss, Jena, Germany).

Results

Patients

Of the 68 patients with squamous cell carcinoma, 55 (82%) were diagnosed as FIGO stage Ib, 10 as FIGO IIa (14%) and 2 as FIGO IIb (3%). Thirty patients (44%) received post-operative radiotherapy due to either tumor positive lymph nodes or a combination of two of the following parameters: depth of infiltration ≥ 15 mm, tumor

size ≥ 40 mm, or the presence of vaso-invasion. Twenty-four patients had lymph node metastases. Twenty-one patients (31%) were HPV16-positive, 18 (27%) were HPV18 positive, 10 (15%) were positive for other HPV types and from 19 patients HPV type was unknown. Prognostic relevance of the findings after microarray analysis was studied with a different group of cervical carcinomas on the tissue array; clinical features from this group are shown in Table 1.

Table 1. Clinicopathological features of patients and tumors and association with SerpinA1 and SerpinA3 staining.

		SerpinA1			SerpinA3		
Patient and tumor		Neg/weak	Strong		Neg/weak	Strong	
characteristics	**N***	n	n	**p-value**	n	n	**p-value**
Age	118						
49 (mean)							
24-87 (range)							
FIGO stage							
1B	94	58	22	0,44	59	19	0,15
IIA/B	24	14	5		17	3	
Lymph node							
Negative	87	53	18	0,43	57	13	0,12
Positive	29	18	9		18	9	
Tumor size							
< 40 mm	67	42	14	0,29	48	7	**0,013**
≥ 40 mm	44	24	13		25	13	
Depth of infiltration							
< 15 mm	66	41	14	0,79	45	9	0,18
≥ 15 mm	51	31	12		31	12	
Vasoinvasion							
No	72	44	13	0,27	44	15	0,65
Yes	39	24	12		26	7	
HPV status ‡							
16	60	39	10	0,41	42	8	**0,008**
18	25	15	8		13	10	
Other	17						
Histology							
Squamous	90	54	24	0,13	59	17	0,97
Adenosquamous**	8	18	3		17	5	
Adeno	19						

N*, number of patients/cervical carcinomas. The total number of reported cases is affected by incidental missing cases. ‡, Association of HPV status with Serpin expression was determined for HPV16 and HPV18. **, Association with histology was performed for adenosquamous/adenocarcinomas versus squamous cell carcinomas. Concerning SerpinA1 and SerpinA3 expression, tumors with less than 2 replicates were excluded from analysis.

HLA class I protein expression and LOH at 6p21.3 in cervical carcinomas

Sixty-eight squamous cell carcinomas were immunostained for HLA class I expression to classify tumors into those with normal, partial and overall loss of HLA class I

expression. Eight of the 68 cases (12%) showed normal expression of HLA class I, 43 cases (63%) showed partial downregulation of HLA class I, and 17 cases (25%) displayed total loss or less than 5% of HLA class I expression (β2m, HLA-A and HLA-B/C negative; Table 2). Since LOH is a major mechanism for loss of HLA class I expression and because positive immunohistochemical staining does not reveal allelic loss, we determined LOH at 6p21.3, the locus of the HLA-A, HLA-B and HLA -C genes. Fifty-six percent (33/59) of the tumors showed loss of 6p21.3 at the HLA class I region (Supplementary Figure 1 and Table 2). Thirty-two of the 68 tumors were selected for gene expression profiling based on HLA class I expression and LOH of the respective locus.

Table 2. LOH of the HLA class I genomic region at 6p21.3 in squamous cervical carcinoma (n = 59) and expression of HLA class I heavy chains (n = 68).

	β2m	HLA-A	HLA-B/C	Total class I	LOH 6p21.3
No expression	13	32	19	17*	8/16
Moderate expression	31	25	29	43**	22/35
Strong expression	24	11	20	8***	3/8

Microsatellite analysis and immunohistochemistry was performed as described in Material and Methods. * Tumors without expression of HLA class I are negative for HLA-A and HLA-B/C. ** Weak expression of HLA-A and/or HLA-B/C or strong expression for HLA-A but not for HLA-B/C or vice versa. *** Strong expression for β2m, HLA-A and HLA-B/C.

Microarray analysis

The 32 selected tumors were divided in three groups: group A, tumors with less than 5% or no HLA class I positive tumor cells (n = 11); group B, tumors with no or partial loss of HLA class I expression and LOH at 6p21.3 (n = 11); and group C, tumors with normal HLA class I expression and no LOH at 6p21.3 (n = 10). Group C included 5 cases with minimal loss, defined as weak expression of HLA-A or HLA-B/C. Statistical analysis revealed no differentially expressed genes between group B and C. Only 15 genes were significantly differentially expressed between group A and B, and 15 genes between group A and C (Supplementary Figure 2). Since no differences in gene expression were observed between the groups with HLA expression (B and C), we combined the samples of group B and C into one group. Overall, 150 genes were significantly differentially expressed between the HLA negative group (group A, n = 11) and the HLA positive group (group B and C, n = 21).

Differences in gene expression and gene ontology

All genes exhibiting statistically significant differences in expression (SSDG) with a defined biological process (n = 105, 68%) were classified by GO terminology. Defense response was the process encompassing most of the SSDGs (Figure 1 and Table 3) and these genes were mainly upregulated in HLA positive tumors (24/27). The program “Global test” was then used to investigate if the defense response as a GO biological process and related subprocesses were significantly different between HLA positive and HLA negative tumors. Indeed, the defense response (which

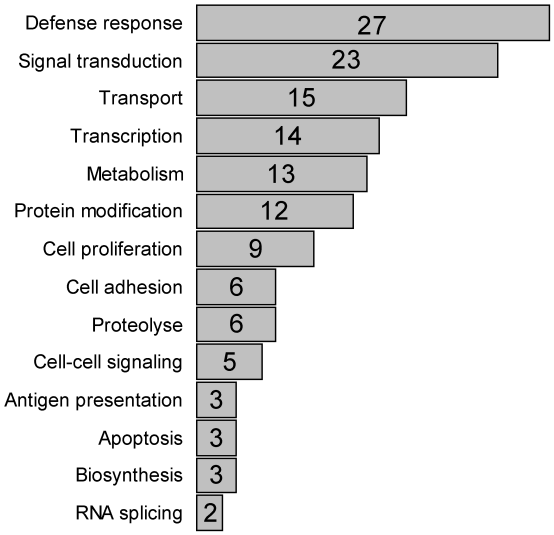


Figure 1. Categorization of genes with statistically significant differences in gene expression (SSDG) into biological processes (using Gene Ontology terms). The numbers behind the bars depict the total number of annotated genes represented on the array for the biological processes.

included 1032 genes, p = 0.0008) as well as the subprocess 'immune response' (925 genes, p = 0.0008) showed significant differences (data not shown). The most prominent subprocesses within the immune response cluster were the "inflammatory response" (256 genes, p = 0.0003, data not shown) and "acute phase response" (33 genes, p = 0.0006, Figure 2). Differences in the amount of inflammatory infiltrate did not influence the differences observed in immune (sub)processes between HLA negative and HLA positive tumors. A correction for the amount of inflammatory infiltrate in the Global test even improved the P-values (data not shown). In the inflammatory response cluster, all SSDGs (11 genes) were upregulated in HLA positive tumors (Table 3). Five of these represented chemokines, such as *CCL3* and *CCL4*. The highest of the SSDGs was *SerpinA3*, which showed an 8.2 fold change in gene expression (Table 3). In the acute phase response (Figure 2), *SerpinA1* and *SerpinA3* were both found to be SSDGs.

qRT-PCR verification of microarray data

Because gene expression differences between the HLA negative and HLA positive group were the most prominent in the inflammatory and acute phase responses, we chose gene products from these processes to validate the microarray results using qRT-PCR. The expression of *SerpinA1*, *SerpinA3* and *CCL4* was significantly increased in the HLA positive tumors (Figure 3). We detected a strong correlation between gene expression data from the microarray and the qRT-PCR experiments ($p < 0.001$).

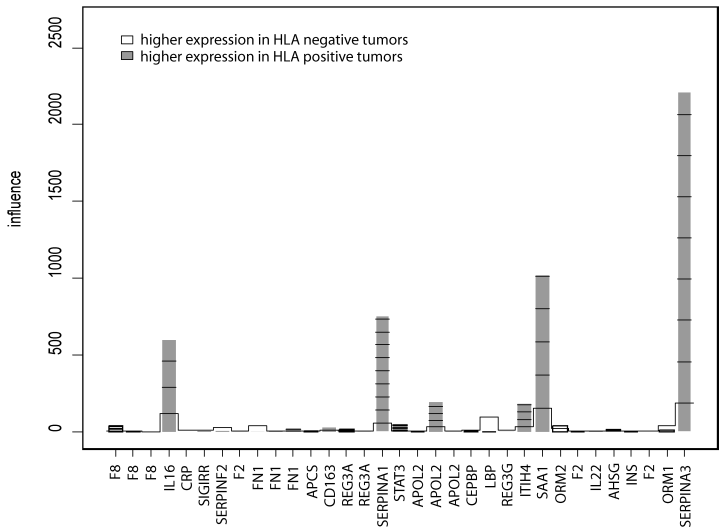


Figure 2. The difference in expression profile of genes involved in the 'Acute Phase response' biological process (defined by Gene Ontology) between tumors positive for HLA expression (grey) and tumors without HLA expression (white). The graph was retrieved from the program Globaltest. The bars indicate the magnitude of difference, and the horizontal black lines in the bars depict standard deviation (SD).

Table 3. Significant differentially expressed genes involved in defense response in HLA-positive compared to HLA-negative tumors.

Gene	Gene Symbol	Fold change	Direction
chemokine (C-C motif) ligand 3	CCL3	3.0	up
chemokine (C-C motif) ligand 3	CCL3L1	2.7	up
chemokine (C-C motif) ligand 4	CCL4	2.7	up
chemokine (C-C motif) ligand 7	CCL7	1.5	up
chemokine (C-C motif) receptor-like 2	CCRL2	1.9	up
chemokine (C-X-C motif) ligand 2	CXCL2	3.6	up
chemokine (C-X-C motif) ligand 3	CXCL3	2.7	up
defensin, beta 106A	DEFB106	1.8	down
FYN binding protein	FYB	1.8	up
ISG15 ubiquitin-like modifier	G1P2	3.4	up
complement factor I	IF	4.3	up
interleukin 18 receptor accessory protein	IL18RAP	2.0	up
interleukin 21	IL21	1.5	up
interleukin 28 receptor, alpha	IL28RA	2.2	down
integrin, beta 2	ITGB2	1.9	up
small conductance calcium-activated channel, subfamily N, member 4	KCNN4	3.1	up
leukocyte immunoglobulin-like receptor, subfamily A, member 2	LILRA2	1.5	up
N-myc (and STAT) interactor	NMI	1.8	up
nuclear receptor subfamily 3, group C, member 1	NR3C1	1.4	up
2',5'-oligoadenylate synthetase 1	OAS1	2.3	up
2'-5'-oligoadenylate synthetase 2	OAS2	2.3	up
2'-5'-oligoadenylate synthetase-like	OASL	3.9	up
serpin peptidase inhibitor, clade A, member 1	SERPINA1	3.6	up
serpin peptidase inhibitor, clade A, member 3	SERPINA3	8.2	up
TIA1 cytotoxic granule-associated RNA binding protein-like 1	TIAL1	1.4	down
toll-like receptor 8	TLR8	1.4	up
GRB2-related adaptor protein 2	GRAP2	1.5	up

Up: upregulated in HLA positive tumors; down: downregulated in HLA positive tumors. The genes shown in bold are genes involved in the inflammatory process (defined by Gene Ontology).

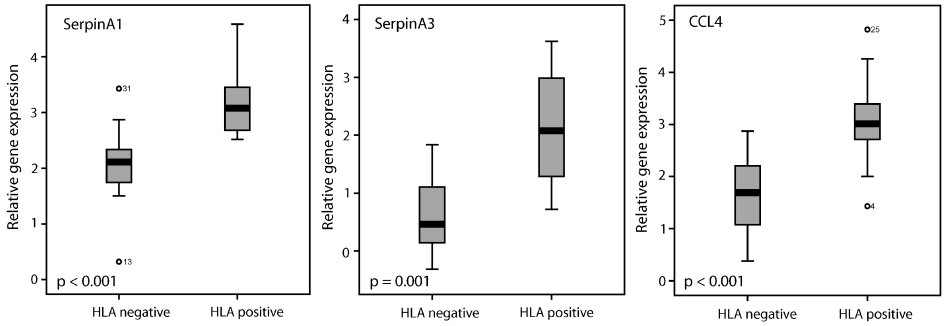


Figure 3. Differences in gene expression between HLA negative and HLA positive tumors. Box plots show differences in gene expression as measured by qRT-PCR of SerpinA1, SerpinA3 and CCL4. P-values were calculated with a Mann-Whitney test.

Immunohistochemical analysis of protein expression

The elevated expression of *SerpinA1* and *SerpinA3* was then verified by immunohistochemistry (Figure 4). Samples with absent or low Serpin expression were combined and compared with the samples that showed strong expression. For both SerpinA1 and SerpinA3, the sum score of protein expression correlated with mRNA expression ($p = 0.04$ and $p = 0.008$, respectively). Similar to the mRNA expression data, a significantly higher expression of SerpinA1 (Fisher's exact, $p = 0.05$) and SerpinA3 (Fisher's exact, $p = 0.02$) protein was observed in the tumors with HLA expression (data not shown). Figure 5 shows immunofluorescence of a tumor that coexpressed HLA class I and SerpinA3 (A) as well as one negative for both HLA class I and SerpinA3 (B).

Association of SerpinA1 and SerpinA3 with clinical parameters and survival

To evaluate the association of Serpin expression with prognostic factors, we immunostained a tissue array that represented 117 tumors. As with the individual slides, the final score for expression of SerpinA1 or SerpinA3 comprised 2 groups: samples with absent/moderate expression, and samples with strong expression. In the tissue array, SerpinA1 expression significantly correlated with HLA expression ($p = 0.03$), whereas SerpinA3 did not show a significant association with HLA expression ($p = 0.14$). Tumor size (≥40 mm) and HPV status significantly correlated with strong SerpinA3 expression ($p = 0.013$ and $p = 0.008$ respectively, Table 1). No significant correlation was observed between HLA expression and HPV type or tumor histotype. HPV type significantly correlated with histotype ($p = 0.02$) but not with tumor size ($p = 0.06$). In addition, a trend for correlation of both SerpinA1 and SerpinA3 with overall survival ($p = 0.068$ and $p = 0.11$, respectively) and disease-free survival ($p = 0.052$ and $p = 0.12$, respectively) was found (data not shown).

Since expression of Serpins may facilitate the immune escape of HLA positive tumors, we next analysed the effect of Serpin expression on survival in cases with normal/partial HLA expression. Strong SerpinA1 expression correlated significantly with poor overall and disease-free survival ($p = 0.035$ and $p = 0.014$, respectively,

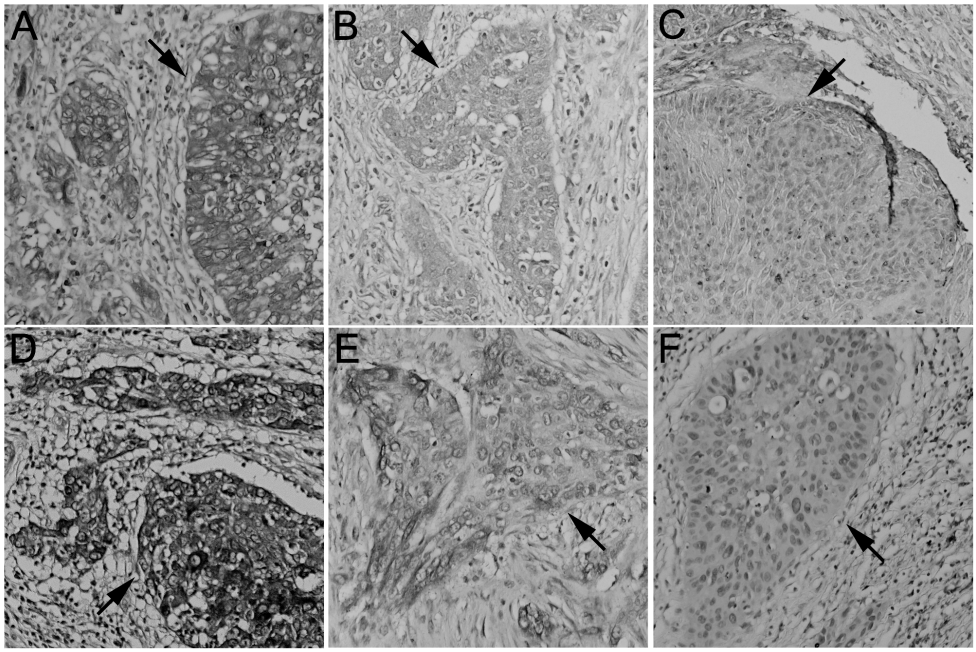


Figure 4. Immunohistochemical analysis of SerpinA1 and SerpinA3 protein expression in cervical carcinomas. Arrows indicate tumor areas. A) Strong staining of SerpinA1, B) weak staining of SerpinA1, C) lack of SerpinA1 staining, D) strong staining of SerpinA3, E) mostly weak staining of SerpinA3 and F) lack of SerpinA3 staining.

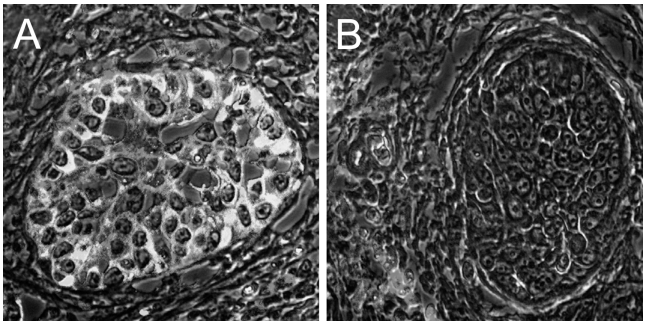


Figure 5. Immunofluorescent staining for SerpinA3 and HLA class B/C. Tumors are depicted with A) positive staining for HLA class I (red, membranous) and SerpinA3 (green, cytoplasmic) or B) no positive staining for HLA class I and SerpinA3, whereas infiltrate cells show positivity.

Figure 6A), and strong SerpinA3 expression showed a correlation with poor overall survival ($p = 0.05$, Figure 6B).

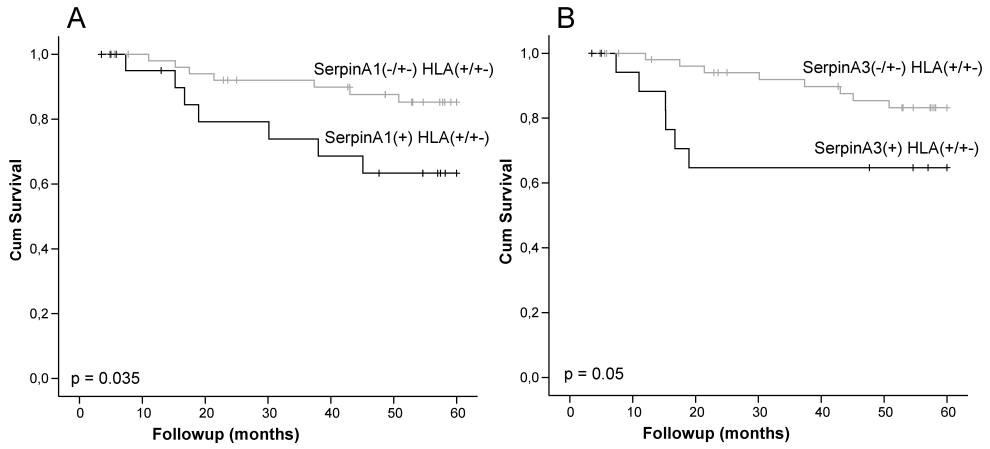


Figure 6. Association between Serpin expression and survival. Kaplan-Meier curves and log rank test for overall survival. Patients with normal/partial HLA expression (+/±) in association with absent/moderate (-/±) or strong (+) expression of A) SerpinA1 and B) SerpinA3.

Discussion

This study shows that loss of HLA class I, as determined by analysis at the genetic and protein level occurs frequently in squamous cell carcinoma. Eighty-eight percent of the tumors showed a downregulation of HLA class I expression, and 56 % of the tumors showed LOH of 6p21.3 at the HLA class I region; this data corresponds to previous results [7].

Gene expression analysis between tumors without or less than 5% of HLA class I positive tumor cells (HLA-) and tumors with normal/partial HLA class I expression (HLA+) showed that “defense response” is the process encompassing most of the SSDGs. No significantly different expression of immunosuppressive cytokines was observed between HLA- and HLA+ tumor groups. In contrast, many genes contributing to inflammatory and acute phase responses, such as *CCL3*, *CCL4*, *CCL7*, *CXCL2* and *CXCL3*, were upregulated in HLA+ squamous cell carcinomas. CCL3 and CCL4 are IFNγ-induced chemokines that initiate the infiltration of macrophages, NK cells and T cells, all of which are cell types that have been associated with anti- and pro-tumor activities. These chemokines can be excreted by stromal cells, immune cells and/or tumor cells.

When we corrected our findings for differences in the extent of inflammatory infiltrate (estimated by visual examination of HE-stained slides) between the groups using the program global test, the immune response (sub)processes remained a significant distinction between HLA+ and HLA- tumors. Thus, the high gene expression profile of inflammatory-related genes in HLA+ tumors is not likely due to a higher number of immune cells.

The pronounced inflammatory gene expression profile of HLA+ tumors could be due to an interaction between immune cells and tumor cells that present viral and/or tumor derived peptides by HLA, leading to activation of the tumor infiltrate and

subsequent release of inflammatory cytokines. Although an inflammatory response is required for the development of an effective T-cell response against tumor cells, it can also contribute to tumor growth and invasion by providing growth factors and releasing proteases [20].

The highest differential expression between the HLA- and HLA+ tumor groups involved the *SerpinA1* and *SerpinA3* acute phase response genes which are upregulated during inflammatory processes. Our study showed that SerpinA1 and SerpinA3 expression at both the mRNA and protein level correlated with the presence of HLA. Both SerpinA1 and SerpinA3 expression showed a trend towards poor prognosis. Previously, high expression of SerpinA1 in tumors was correlated with poor prognosis in different types of adenocarcinomas [21,22].

The mechanisms by which SerpinA1 and SerpinA3 promote tumorigenesis and tumor growth remain unclear. In colorectal and lung adenocarcinomas, SerpinA1 expression correlated with the invasive and metastatic capacity of tumor cells [23,24]. Also, an immunosuppressive role for SerpinA1 as strong inhibitor of NK cytotoxicity *in vitro* has been reported [25]. Consistent with this, SerpinA1 was shown to be one of the most potent *in vitro* inhibitors of granzyme B [26], a trypsin-like serine protease by which cytotoxic T cells (CTL) and NK cells mediate apoptotic death of target cells. SerpinA3 expression has been detected in various tumors, including gastric and hepatocellular carcinomas [27,28]. Chymotrypsin, a target of SerpinA3, can induce apoptosis in leukaemia and hepatoma cells *in vitro*, and SerpinA3 inhibits apoptosis of hepatoma cells [29,30]. Thus, although a precise role for SerpinA1 and SerpinA3 in cancer remains to be elucidated, these proteins could be involved in tumor invasion or inhibition of apoptosis.

We recently used the tissue array described in the present study to show that partial loss of HLA, compared to complete loss or normal HLA expression, correlated with disease progression [31]. Low expression of HLA class I could assist the tumor cell in escaping from both CTL and NK cell-mediated killing [32]. Since SerpinA1 and SerpinA3 expression did not show a significant correlation with survival, the influence of these proteins on carcinogenesis is likely dependent on other factors. As SerpinA1 and SerpinA3 expression was most prominent in HLA+ tumors, we investigated whether Serpin expression may contribute to disease progression in HLA+ tumors. We observed that strong expression of both SerpinA1 and Serpin A3 in association with normal/partial HLA expression correlated with poor prognosis. Thus, immune evasion in HLA+ tumors could be attributed in part to factors such as SerpinA1 and SerpinA3 that possibly mediate an invasive and/or anti-apoptotic effect, thereby contributing to a more aggressive behaviour of the tumor.

In conclusion, here we show that the gene expression profile of HLA+ tumors differs from that of HLA- tumors with respect to expression of genes involved mainly in inflammatory and acute phase immune responses. Elevated expression of SerpinA1 and SerpinA3 in HLA+ tumors was confirmed by qRT-PCR and immunohistochemistry. Furthermore, strong expression of either SerpinA1 or SerpinA3 associated with HLA expression is detrimental for survival.

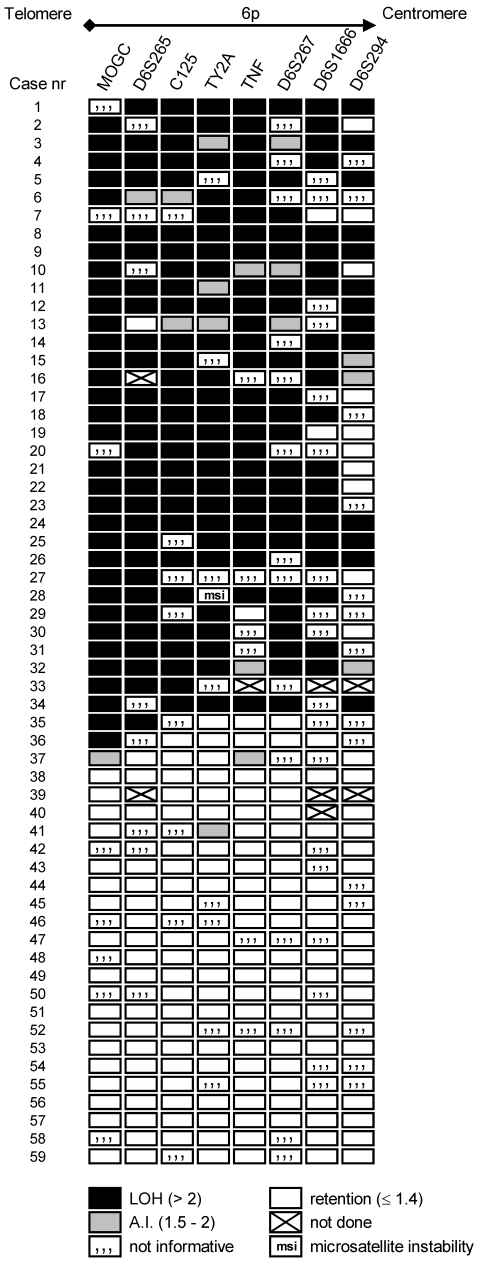
Acknowledgements

This study was supported by the Dutch Cancer Society, grant no. RUL2001-2465. The study was performed within the Centre for Medical Systems Biology, a centre of excellence supported by the Netherlands Genomics Initiative/ Netherlands organization for Scientific Research (NWO).

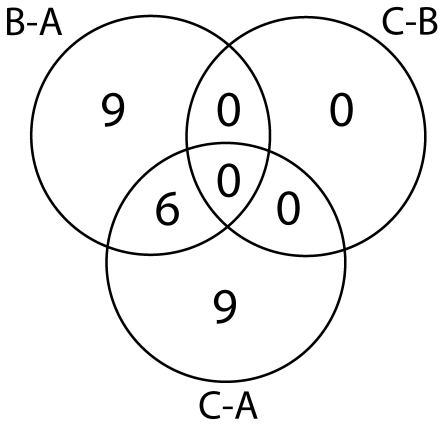
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Supplementary Figure 1. Microsatellite analysis at 6p of 59 squamous cell carcinomas. As described in Materials and methods, microsatellite analysis was performed on flow-sorted tumor cell subpopulations and frequently approached 100% signal reduction in one of the alleles. A.I.: allelic imbalance.



Supplementary Figure 2. Number of significant differences in gene expression between tumors with overall loss of HLA class I (A), tumors with partial loss of HLA class I (B) and tumors with no or minimal loss of HLA class I expression (C). Statistical analysis was performed by a nested F-analysis as described in Materials and Methods. The Venn diagram demonstrates 15 genes to be differentially expressed between group A and B and 15 genes between group A and C of which 6 genes are shared. No differences in gene expression were observed between group B and C.

3

Lack of TNFα mRNA expression in cervical cancer is not associated with loss of heterozygosity at 6p21.3, inactivating mutations or promoter methylation

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Molecular Immunology. 2008; 45(1): 152-159

Infection with oncogenic human papillomavirus is considered to be the major etiologic event for cervical cancer. Tumor necrosis factor α (TNFα), a proinflammatory cytokine, may be involved in orchestrating an antitumor immune response against human papillomavirus expressing cervical cancer cells. Hence, loss of TNFα could be advantageous for tumor cells to escape immune clearance. The aim of our study was to investigate *TNFα* gene expression and (epi)genetic characteristics associated with the loss of *TNFα* expression in cervical cancer. To this end we examined *TNFα* expression, loss of heterozygosity (LOH) at 6p21.3, the locus of *TNFα*, mutational status of the *TNFα* locus, loss of the *TNFα* promoter variant 2 allele and CpG hypermethylation of the *TNFα* promoter. RNA in situ hybridization showed absence of *TNFα* expression in 45% of 63 tumors. LOH occurred in 57% and was not concordant with absence of *TNFα* mRNA. No mutations in the *TNFα* gene were identified in 15 cases deficient in *TNFα* expression exhibiting LOH. Furthermore, lack of *TNFα* expression did not correlate with promoter methylation. In conclusion, *TNFα* mRNA expression is absent in nearly half of the cervical tumors analyzed. Neither promoter methylation nor genetic causes for lack of expression are evident.

Introduction

Human papillomaviruses (HPV) are DNA viruses that induce epithelial cell transformation during the course of infection [1]. Infection with oncogenic HPVs can result in precursor lesions, i.e. cervical intraepithelial neoplasia (CIN), that usually regresses when an appropriate host immune response is activated against the virus [2,3]. Persistent viral infection is required for neoplastic progression, and failure of virus clearance has been attributed to a poor immunological response [4].

In an immunocompetent host, inflammatory cytokines play an important role in the immune surveillance mechanisms against cervical cancer [5]. One of these cytokines is tumor necrosis factor α (TNFα). TNFα plays a role in the killing of tumor cells by activated macrophages [6,7] and in the generation of cytotoxic T-cells that are able to lyse cervical cancer cells [8]. An underlying cause of a failing antitumor immune response in the case of cervical carcinoma was suggested to be a locally altered cytokine environment with an increase in immunosuppressive cytokines and a decrease in proinflammatory cytokines such as TNFα [9,10].

Indeed, in cervical cancer cell lines no *TNFα* mRNA expression was detectable [11]. Loss of *TNFα* expression can either be caused by genetic silencing due to gene mutation and deletion of the other functional allele, or by epigenetic silencing due to lack of transcriptional activation or hypermethylation of the gene promoter for example. Several studies have identified a high incidence, approximately 40% of loss of heterozygosity (LOH), a well-known mechanism of tumor suppressor gene (TSG) inactivation, at 6p21.3-p25, where the locus of *TNFα* is located [12,13]. However, although loss of HLA class I alleles has been amply reported, no 'classical' TSG has yet been discovered in the 6p region. An alternative mechanism that can influence the expression of TNFα is the presence of single nucleotide polymorphisms (SNPs) in the promoter of the gene. The frequently occurring *TNFα* promoter variant 2 (*TNF2*) allele of the -308G/A *TNFα* gene promoter polymorphism, has been associated with increased *TNFα* gene expression [14,15]. If *TNFα* is a TSG one can predict that LOH would preferentially affect the *TNF2* allele. A limited number of studies have investigated the association of *TNFα* promoter polymorphisms with cervical cancer [16-19], however the results were inconsistent. Thus a role for *TNFα* promoter polymorphisms in the carcinogenesis of cervical cancer remains unresolved. Epigenetic silencing by means of CpG methylation in the promoter has been shown in HeLa, monocytes, lymfocytes granulocytes and leukemic cells [20,21], but has not been investigated in solid tumors.

To elucidate the role of TNFα in cervical cancer carcinogenesis we have addressed the expression status of *TNFα* mRNA as well as genetic and epigenetic causes for low or absent mRNA expression in cervical cancer.

Materials and methods

Patient material

Sixty-five archival formalin-fixed, paraffin-embedded cervical carcinomas and one normal cervical sample were obtained from the tissue bank of the Department of

Pathology (LUMC, Leiden, The Netherlands).

Preparation of TNFα probe & RNA in situ hybridization

RNA in situ hybridization (RISH) was performed as previously described [22]. Tumor sections were hybridized overnight with 100 ng/ml of *TNFα* anti-sense and sense digoxigenin-labeled cRNA probe, at 62°C with 4x standard saline solution citrate. Normal tonsil tissue served as a positive control. All cases were hybridized with a probe for β-actin to ensure the RNA quality and fixation of the tissue samples. RISH was given an intensity and a percentage scores, based on intensity of cytoplasm positive staining and proportion of cells staining respectively [23].

Genomic DNA isolation from flow-sorted tumor cell subpopulations

Formalin-fixed, paraffin-embedded cervical carcinomas were trimmed if necessary to remove normal epithelium and CIN. The remaining tumor tissue was dissociated, stained for keratin, vimentin and DNA as previously described [24]. The vimentine-negative keratin-positive (V-K+) fraction, which represents the epithelial cell subpopulation, and vimentin-positive keratin-negative (V+K-) fraction, consisting of normal cells, were flow sorted using a FACS Vantage (BD Biosiences, San Jose, CA). From seventeen multiploid samples two V-K+ fractions with differing ploidy were flow-sorted. DNA was extracted as previously described [25].

Microsatellite analysis

Eight fluorescein-labeled primer pairs of microsatellite markers comprising MOGC, D6S265, C125, TY2A, TNF, D6S273, D6S1666 that span 6p21.3, the locus of TNFα, and D6S294 (Genome Database, http://www.gdb.org) at 6p12.1, were used. PCR, electrophoresis and analysis was performed as described previously [26]. Comparing normal and tumor DNA, a reduction of 80% of one allele was assigned as complete LOH. The range for incomplete LOH was allocated between more than 50% and less than 80% of reduction. A reduction of 25% or more of one allele was assigned as allelic imbalance (A.I.).

Mutation analysis

Primers designed for amplification of the entire exon sequence of *TNFα*, applicable for paraffin material as template, are listed in Table 1. The following PCR conditions were used: 10 min at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds extension at 72°C.

TNFα promoter gene polymorphism

To reveal a polymorphic variant of the *TNFα* gene, the *TNF2* allele, which is characterized by a guanine to adenine polymorphism at position -308 in the *TNFα* promoter, primers containing a single base-pair mismatch adjacent to the polymorphic site were used to introduce a restriction site into the wild-type nucleotide sequences after amplification [27]. The amplified DNA fragment appears as a 117 bp band, which when digested with NCOI restriction enzyme, produces a 97 bp and a 20 bp band in case of *TNF1* (-308G), while its homologue *TNF2* (-308A) is undigested and remains a single band of 117 bp.

Table 1. Primers used in PCR and sequencing of the TNFα gene.

Primer	Size (bp)	Sequence
Exon 1.1 F	151	5'-ATACTGACCCACGGCTTCAC
Exon 1.1 R		5'-TTCTCCGACTCCTTGTTCGT
Exon 1.2 F	182	5'-TGCTTGTTCCTCAGCCTCTT
Exon 1.2 R		5'-GAGAGAGATGGGATGGGTGA
Exon 2 F	216	5'-TGACTCCCTCGATGTTAACC
Exon 2 R		5'-GATGGAGGTGAAAGTAGGG
Exon 3 F	218	5'-GATGGAGGTGAAAGTAGGG
Exon 3 R		5'-GAACTTGGAGGGCTAGGAT
Exon 4.1 F	202	5'-AACCGACATGGCCACACTG
Exon 4.1 R		5'-CTCCCAGGTCCTCTTCAAG
Exon 4.2 F	176	5'-GGCGTGGAGCTGAGAGATAA
Exon 4.2 R		5'-AACCTCCTCACTGCCATCAA
Exon 4.3 F	185	5'-AACCTCCTCTCTGCCATCAA
Exon 4.3 R		5'-TCTGGGCAGGTCTACTTTGG
Exon 4.4 F	180	5'-CTATCTGGGAGGGGTCTTCC
Exon 4.4 R		5'-CAACCTTCCCAAACGCCTC

Cell lines and cultures

Human cervical cancer cell lines, HeLa, CaSki (ATCC, Rockville, MD), CC11- and CC8 [28] were maintained in RPMI 1640 as described [29]. Normal primary cervical epithelial cell culture (NPE) was prepared as described [11]. To stimulate *TNFα* expression in peripheral blood mononuclear cells (PBMC), Ficoll-isolated PBMC were cultured o.n. with 2 ml phorbol 12-myristate 13-acetate (PMA) and 3 ml Ionomycine.

Quantitative real-time PCR (qRT-PCR)

RNA isolation, cDNA synthesis, PCR and normalization were done as described [29]. Primer sequences are: 5'-CCCAGGGACCTCTCTCTAATCA-3' and 5'-CTCAAGCTGAGGGGCAGCT-3'. Intron-spanning primers localized on exons 2 and 4, starting at bp position 967 of the gene, were used to amplify a 116-bp fragment.

Methylation

The bisulphite method was applied for DNA methylation analysis using the EZ DNA Methylation Kit (ZYMO Research, Orange, USA) according to the manufacturer's protocol. To determine which CpGs in the promoter and exon 1 are relevant for transcription silencing, up to 1500 bp upstream of the transcription start was sequenced (Genbank accession number no. M16441) of the mentioned cell cultures using primers listed in Table 2. Of the sorted cells only the relevant CpG sites in the promoter was sequenced using primer pair 6.

Results

TNFα expression in cervical carcinomas

To investigate whether *TNFα* expression is lost in cervical cancer cells, gene expression was studied in 62 tumors by RISH. Twenty-eight (45%) tumors showed

Table 2. Primers used for detection of methylated cytosine by sequencing.

Primer	Annealing temp (°C)	Size (bp)	Sequence
Promoter 1 F	57	339	5'-AGGGTTTTAGAAGGTAGGGAAA
Promoter 1 R			5'-AGTTAGGGTTTGGAGGGGTG
Promoter 2 F	52	359	5'-TTAGGTGAGGTTGTTAGATTG
Promoter 2 R			5'-GAGAAGTGAGAGTTTTATGAA
Promoter 3 F	55	356	5'-GTTTTTGGGTTAGAATGAAAGA
Promoter 3 R			5'-AAATGGAGGTAATAGGTTTTG
Promoter 4 F	55	200	5'-GTTTGGAAGTTAGAAGGAAAT
Promoter 4 R			5'-TTTTTGATGTTTGTGTGTTTTT
Promoter 5 F	55	450	5'-TTTTTGTATTTTGTTTGGAAGTT
Promoter 5 R			5'-GAGGATTAGTTAAGAGGGAGAGA
Promoter 6 F	60	265	5'-GGTATTTTTGATGTTTGTGTGTTTT
Promoter 6 R			5'-GAGGATTAGTTAAGAGGGAGAGA
Exon 1 F	55	362	5'-TAGAGGATTAGTTAAGAGGGAGAG
Exon 1 R			5'-TGAGTGTTTGGTTAGTTTTTATTT

no expression, 21 (34%) had intermediate expression and 13 (21%) demonstrated high expression of *TNFα* in tumor cells (Figure 1). All tumors were positive for *TNFα* expression of cells in the tumor stroma. Most likely these cells are inflammatory cells. Twenty-five of 29 tumors (86%) displayed expression for *TNFα* in normal epithelium, adjacent to the tumor (Figure 1). This implies a decline in *TNFα* expression of epithelial cells during development of cervical cancer. No association of *TNFα* expression by the tumor cells with clinicopathological parameters (Table 3) or survival (data not shown) was observed.

LOH at 6p

Subsequently we investigated whether lack of *TNFα* expression was associated with LOH. Sixty tumors were eligible for microsatellite analysis. High allelic ratios were frequently observed, mostly ranging from 5-10 and often higher than 10, representing complete LOH (Table 4 and Figure 2A). LOH at the *TNFα* locus was apparent in 34 cases (57%). Usually, most of the markers spanning the 6p21.3 region showed LOH (Figure 2A and 2B). Of these cases the marker near the centromere

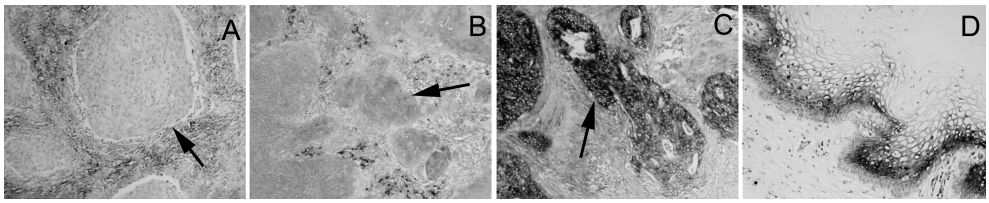


Figure 1. *TNFα* mRNA expression in cervical cancer and normal cervical squamous epithelium (´ 100). mRNA expression was measured by RISH as described in Materials and methods. The arrows point to tumor areas. A) Squamous cell carcinoma showing no *TNFα* expression, B) Squamous cell carcinoma showing low *TNFα* expression, C) Adenocarcinoma showing high *TNFα* expression and D) Normal cervical squamous epithelium showing *TNFα* expression.

Table 3. Summary of clinicopathological features of patients and tumors and association with *TNFα* expression.

Patient and tumor characteristics	Outcome	N*	p-value
Age	46 (mean)	65	
	26-72 (range)		
FIGO	1B	47	0,232
	IIA/B	16	
Lymph node metastasis	No	45	0,624
	Yes	20	
Tumor size	≥ 40 mm	39	0,233
	< 40 mm	26	
Depth of infiltration	≥ 15 mm	29	0,921
	< 15 mm	36	
Vascular space involvement	No	26	0,931
	Yes	32	
Parametrial invasion	No	61	0,868
	Yes	3	
HPV status ‡	16	18	0,134
	18	20	
	Other	9	
Histology	Squamous	49	0,506
	Adenosquamous	7	
	Adeno	8	

N*, number of patients/cervical carcinomas. ‡ Only data for cervical carcinoma samples with a determined HPV type were included, other subtypes included HPV31 (n=2), HPV33 (n=1), HPV45 (n=1), HPV51 (n=1), HPV52 (n=1), HPV56 (n=1), HPV59 (n=1) and HPV67 (n=1). The number of reported cases is affected by incidental missing cases. P-value depicts association of the particular clinical parameter with TNFα expression.

Table 4. Summary of microsatellite analysis results for *TNFα*, arranged according to level of gene expression.

	n (%)			
	Total	**TNFα absent***	**TNFα intermediate†**	**TNFα high‡**
Retention	22 (39)	10 (45)	9 (41)	3 (14)
A.I. 2-5	10 (18)	7 (70)		3 (30)
A.I. 5-10	25 (44)	11 (44)	7 (28)	7 (28)

*Absent *TNFα* indicates a score of 0, †intermediate *TNFα* a score of 2-5 and ‡high *TNFα* expression to a score of 6-8 (De Ruiter). A.I., allelic imbalance.

was lost in 22 (39%) of cases suggesting that in those cases the whole chromosome arm may be lost. No association was observed between expression of *TNFα* and the occurrence of LOH (Table 4). Eight out of 14 multiploid tumors displayed LOH with an A.I. of more than 2 for both diploid and aneuploid fractions (Figure 2A). An example of LOH in both diploid and aneuploid fractions of a tumor is shown in Figure 2C.

Mutation analysis of the TNFα gene in cervical samples lacking TNFα expression

To investigate if in combination with LOH, an inactivating mutation or deletion of the

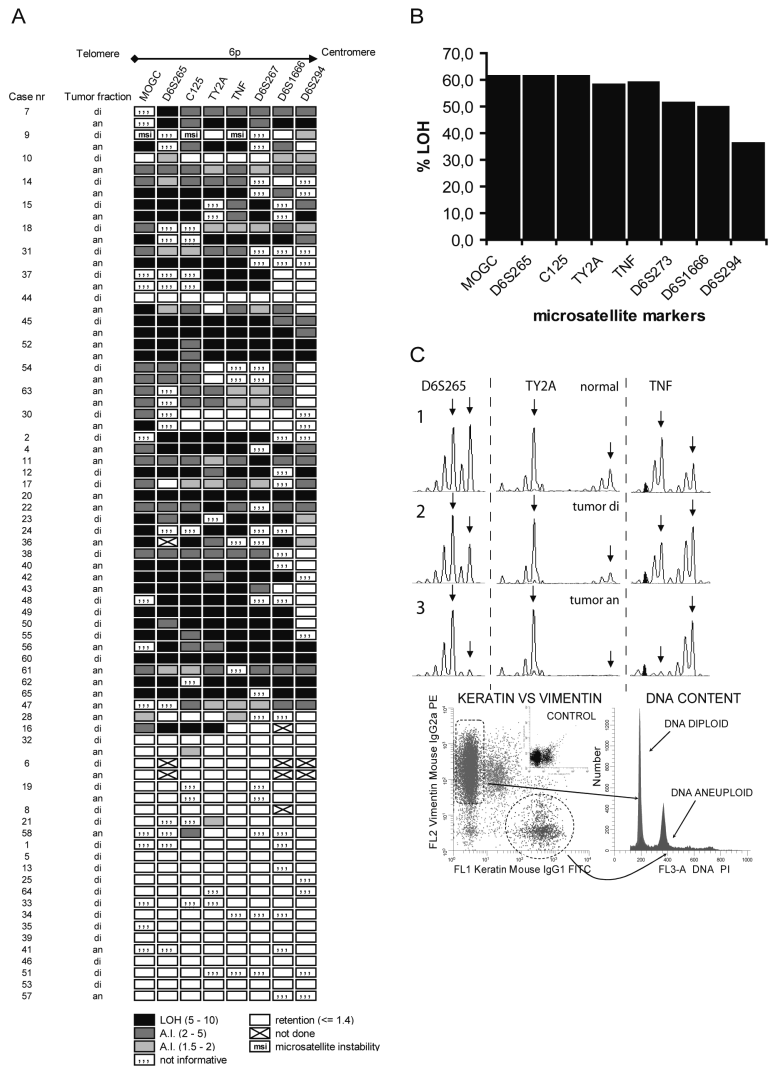


Figure 2. LOH at 6p21.3. A) Microsatellite analysis at 6p of 63 samples, as described in Materials and methods. B) The percentage of LOH for each marker. C) LOH profile of tumor 31. This tumor contained a diploid and aneuploid subpopulation. The first panel represents the normal (V+K-) subpopulation of the tumor that is heterozygous for the three markers: D6S265, TY2A and TNF. The arrows point to the different alleles. The black peak, visible in the graphs of TNF, is a size marker. The second panel, represents the diploid tumor (V-K+) subpopulation with an allelic imbalance (A.I.) of ~2 at 6p, depicting an apparent decrease in one of the alleles for the 3 markers. The third panel shows complete loss of the allele for the three markers in the aneuploid tumor cells. The two graphs below illustrate the flow sorted subpopulations of the multiploid tumor. In green, the V+K- fraction, consisting of normal cells, is depicted (left graph). In red, the K+V- fraction, representing the tumor cells, is visible. As shown in the right graph, most of the tumor cells are aneuploid and a few are diploid, whereas the normal cells are all diploid.

TNFα gene is the underlying cause for lack of mRNA expression, and *TNFα* thus behaves as a classical TSG, 15 out of 18 tumor samples with LOH of 6p21.3 and without *TNFα* expression were examined for an aberration in the exon sequences of *TNFα*. The complete coding sequence was analyzed but no mutations or deletions were found (data not shown).

Preferential loss of TNF2

An alternative mechanism to reduce *TNFα* expression is loss of the *TNF2* allele. To determine if the 'high-producer' *TNF2* allele is preferentially lost in cases with LOH, an allele-specific restriction-digest was performed (Figure 3). The prevalence of the polymorphism was 38% (24/63). Homozygosity for the *TNF2* allele was observed in 2 samples. Thirteen out of 38 (34%) cases with LOH at 6p21.3 were heterozygous for the *TNF2* allele. From these 13 cases, 7 cases had lost the *TNF2* allele and 6 retained the normal allele (*TNF1*). Therefore, no preferential loss of the *TNF2* allele was shown. In addition, no significant association between the expression level and the presence of *TNF2* (Fisher's exact test, p=0.475) was observed.

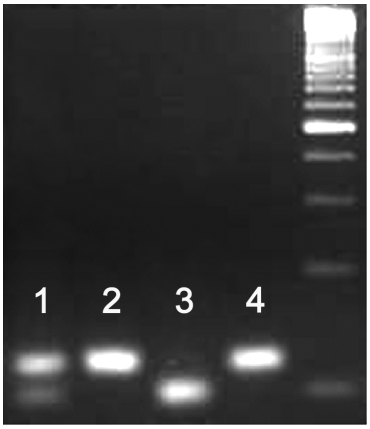


Figure 3. Detection of the *TNF2* allele by NCO 1 restriction digest. *TNF2,* a polymorphism within the *TNF*α gene promoter, was identified by PCR and subsequently sequence-specific restriction, as described in Material and methods. Lanes 1 to 4 represent (un)digested PCR products from the same patient, who is heterozygous for *TNF2*. Lane 1 shows the PCR product from V+K- (normal) cell DNA after NCO 1 restriction digest, revealing a 97 and a 117 bp band representing the normal allele, *TNF1*, and *TNF2*, which remains undigested. Lane 2 shows the same PCR product before digestion. Lane 3 depicts the PCR product from V-K+ (tumor) cell DNA after NCO 1 restriction digest, revealing only a 97 bp band, representing *TNF1*, indicating that *TNF2* is lost in the tumor. Lane 4 depicts the latter PCR product before digestion.

Methylation of CpGs

An alternative to genetic silencing of *TNFα* expression might be methylation of the *TNFα* promoter and exon 1. To identify the crucial CpG islands for transcription, expression of *TNFα* was associated with the methylation status of CpG islands *in vitro* using cervical cancer cell lines (mutation-analysis showed no aberrations in

the *TNFα* sequence), PMA stimulated PBMC and NPE. PBMC and NPE showed expression of *TNFα*, as measured by real-time quantitative PCR (data not shown), and showed either absence of methylation or hemimethylation of CpGs located in a promoter region which is 200 to 400 bp upstream of the transcription start in a transcription box rich area (Figure 4). In addition, all CpGs present in exon 1 showed absence of methylation. In the cell lines (HeLa, CaSki, CC8 and CC11-), these CpGs were methylated and corresponded with absence of *TNFα* expression, implying a role for these CpGs in transcriptional silencing (Figure 4).

In 14 flow-sorted tumor samples, the methylation status of the respective CpGs in the promoter was determined. In paraffin-sorted samples exon 1 was excluded from analysis because of echoes in the sequence at sites of long T-stretches, after conversion of Cs to Ts, making the sequence at these places unreadable. Only 2 out of 9 samples without expression showed methylated CpGs in the promoter (Figure 4).

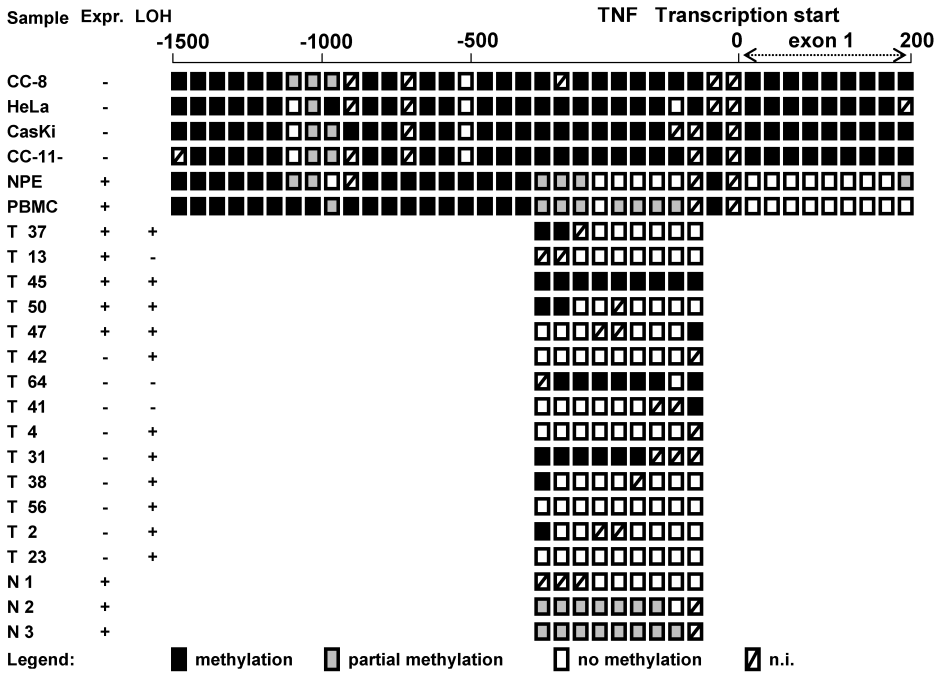


Figure 4. CpG methylation profile of the promoter and exon 1 of *TNFα*. In cell lines, NPE and PMA stimulated PBMC, the methyaltion state of CpGs is shown. The line above depicts the relative position of the CpG islands in the promoter region (bp position -1500 to 0) and exon 1 (bp position 0 to 200). All CpGs present in exon 1 and the promoter region, from bp position -1500 up to 0 (transcription start), are depicted in successive order. CpG methylation of a part of the promoter is shown for tumor (V-K+) samples in parallel with expression and LOH status. Three normal cervical epithelium samples derived from paraffin-embedded tissues were included. N = normal, T = tumor, Expr. = expression, n.i. = not informative.

Discussion

The present study shows lack of *TNFα* expression in 45% of cervical cancers studied. From the tissue samples, 25 out of 29 cervical tumors (86%) displayed expression for *TNFα* in adjacent normal epithelium. These results confirm gene expression of *TNFα* in normal cervical epithelial cells [11,30] and the absence of *TNFα* expression in a substantial number of cervical tumors [22].

Subsequently, we addressed the question whether *TNFα* might be a classical TSG in cervical cancer. The use of highly enriched tumor samples for molecular analysis, derived from FACS-sorted cells, ensures reliable LOH data and explains the relatively high (60%) frequency of LOH at 6p21.3, found in our study. Most tumors showed LOH in both diploid and aneuploid fractions, suggesting that LOH of 6p occurs before ploidy changes arise and therefore is an early event in carcinogenesis. No association between loss of *TNFα* through LOH and absence of expression was found suggesting that there is no selective pressure on the *TNFα* gene. However, since LOH was detected in a part of the tumors deficient in *TNFα* expression, 15 of these tumors were subjected to mutation analysis. No mutation or deletion was observed as a second hit to inactivate the *TNFα* gene. To our knowledge, no genetic inactivation of *TNFα* has been reported so far.

Subsequently, we hypothesized that low *TNFα* expression, as found in 33% of the tumors, may be due to loss of the *TNF2* allele since this allele has been associated with a higher level of *TNFα* expression [31,32]. Recently, Duarte et al. showed a higher frequency of the *TNF2* allele in cervical cancer patients, suggesting that the 'high producer' *TNF2* allele would be associated with an increased risk for the development of cervical cancer [33]. However, other studies did not observe an association of the *TNF2* allele with cervical cancer [17-19]. Our data do not support a role for *TNF2* in cervical cancer since there is no preferential loss of the *TNF2* allele and no evidence for a role of *TNF2* in enhancing transcription because no association with expression was observed.

As for epigenetic ways of silencing transcription, an association between expression and CpG methylation pattern of *TNFα* in HeLa, CaSki, CC8, CC11-, NPE, PBMC and cervical epithelium, was apparent. The relevant CpG islands identified, which differed in methylation profile between samples expressing *TNFα* and samples lacking *TNFα* expression, corresponded with previous data [20]. The respective *TNFα* promoter region contains many transcription factor binding sites [34]. Of the 9 clinical samples without expression, only 2 showed methylation in the promoter, implying that other factors must play a role in regulating transcription, e.g. histone modifications [35] or the presence of specific transcription factors [36].

In conclusion, *TNFα* mRNA expression is absent in nearly half of the cervical tumors analyzed. In spite of comprehensive analysis, LOH accompanied by mutation in the remaining allele or methylation of CpG islands in the promotor region or exon 1, both major mechanisms that are involved in altering gene expression, did not appear to play a role in reducing *TNFα* mRNA expression in cervical cancer since cervical cancer samples lacking *TNFα* gene expression did not show a mutation or a consistent methylation profile in combination with LOH, nor did preferential loss of

the 'high producer' *TNF2* occur.

Acknowledgements

We thank S. Uljee for technical assistance, Dr. H. Baelde for advice and assistance with RISH, and Dr. C. Cornelissen for critically reviewing the manuscript. The study was supported by the Dutch Cancer Society, grant no. RUL2001-2465.

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4

Substantial changes in gene expression of Wnt, MAPK and TNFα pathways induced by TGF-β1 in cervical cancer cell lines

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Carcinogenesis. 2005; 26(9): 1493-1502

Transforming growth factor-beta 1 (TGF-β1) is a potent inhibitor of epithelial cell proliferation. During the development of cervical carcinoma however, an increase in production of TGF-β1 is accompanied by decreased sensitivity for the growth-limiting effect of TGF-β1. TGF-β1 has an anti-proliferative effect on cells of the immune system and thus can be advantageous for tumor progression. The aim of the present study was to determine the effect of TGF-β1 on mRNA expression profile of genes in pathways involved in cell growth and cell death, in cervical carcinoma cell lines with different sensitivity to TGF-β1. For this purpose, we have investigated changes in gene expression in TGF-β1 stimulated cervical cancer cell lines with high (CC10B), intermediate (SiHa) and low (HeLa) sensitivity to the anti-proliferative effect of TGF-β1, at timepoints 0, 6, 12 and 24 hrs. Microarray analysis, using Affymetrics focus arrays, representing 8973 genes, was used to measure gene expression. In our study novel target genes involved in tumor necrosis factor alpha (TNFα), mitogen-activated protein kinase (MAPK) and wingless type (Wnt) pathways in response to TGF-β1 were found. Substantial differences in gene expression between TGF-β1 sensitive and insensitive cell lines were observed involving genes in TNFα, MAPK, Wnt and Smad pathways. Since these pathways are implicated in cell proliferation and cell death, these pathways may play a role in determining the overall sensitivity of a cell to TGF-β1 induced cell growth inhibition. The results were subsequently validated by quantitative real-time PCR. Increased resistance to TGF-β1 induced cell growth inhibition was correlated with an elevated production of TGF-β1 by the cell lines, as measured by ELISA. TGF-β1 production did not inhibit cell growth, since blocking TGF-β1 protein by anti-TGF-β had no effect on cell proliferation. TGF-β1 excretion by tumor cells more likely contributes to paracrine stimulation of tumor development.

Introduction

TGF-β1 plays an essential role in cellular processes such as proliferation, differentiation, embryonic development, angiogenesis, wound healing and inhibition of epithelial cell growth. During cancer development however, the majority of tumor cells become either partly or completely resistant to TGF-β1 growth inhibition and TGF-β1 induced apoptotic responses [1,2]. In cervical carcinoma, extracellular levels of TGF-β1 are increased in late stage of malignancy [3,4], thereby contributing to invasion, metastasis and inhibition of host-tumor immune responses [5-7].

Defective TGF-β1 signaling, due to inactivating mutations of TGF-β1 receptors (TβR1) and Smad signaling molecules, has been reported in cervical carcinoma [8,9]. However, only a few cervical cancers showed mutations in TGF-β1 receptors or Smad [10,11] and this does not explain the loss of growth inhibition in the majority of cervical cancers.

Hence, other mechanisms, besides functional loss of TβR1, TβRII and Smad, are likely to exist. In different types of cancer a defect in the TGF-β1 pathway downstream of Smad signaling has been suggested [12] and crosstalk of TGF-β1/Smad signaling with other pathways have been demonstrated [13,14]. MAPK, phosphoinosytol-3 kinase (PI3K) and Rho signaling via TGF-β1, with or without crosstalk with Smad signaling, have shown to be involved in inhibition of apoptosis in cancer cells, invasion and metastic processes [13,15]. In addition, crosstalk between TGF-β1 and Wnt [16,17], TNFα [18], interferon γ (IFNγ) [19] and epidermal growth factor (EGF) [20] have been demonstrated, which may influence tumorigenesis. This suggests that differential regulation of TGF-β1 signaling, other than attenuation of TGF-β1-Smad signaling by reduced expression or functional loss of Smad pathway components, may determine the magnitude of the TGF-β1 response.

Only a few studies investigated large-scale gene expression changes after stimulation with TGF-β1 in cancer cells. These studies focused primarily on epithelial to mesenchym transition (EMT) processes and did not address loss of cell growth inhibition [21,22]. The aim of the present study was to determine the effect of TGF-β1 on mRNA expression profile of genes in pathways involved in cell growth and cell death, in cervical carcinoma cell lines with different sensitivity to TGF-β1. For this purpose we used cDNA microarrays to measure differences in gene expression between cervical cancer cell lines with low (HeLa), intermediate (SiHa) and high (CC10B) sensitivity to TGF-β1, respectively.

Materials and methods

Cell lines and cultures

Human cervical cancer cell lines, HeLa, SiHa (ATCC, Rockville, MD) and CC10B [23] were maintained in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% fetal calf serum (FCS), streptomycin and penicillin at 37°C in a humid atmosphere containing 5% CO_2.

Growth inhibition study

Cells were plated onto 24-well plates (Greiner, Frickenhausen, Germany) at a density of 3-5 x 10^3 cells/well in 0.5 ml culture medium. The following day, cells were incubated with different amounts of human recombinant TGF-β1 ranging from 0.0001-1 ng/ml (SIGMA, Saint Louis, USA). Each day recombinant TGF-β1 was added to the cells over a period of 6 days. Cells were counted every 2nd day in triplicate using a CASY1 cell counter (Schärfe System, Reutlingen, Germany). Growth kinetics experiments were repeated at least two times. To examine growth arrest, induced by a cell's own produced TGF-β1, TGF-β antibody (2G7) in a concentration ranging from 0.1 to 10 μg/ml was added to the cell culture.

TGF-β1 ELISA assay

For quantification of TGF-β1 protein in cell culture supernatant, the Quantikine Immunoassay (R&D Systems Europe, Abingdon, UK) was performed according to the manufacturer's instructions. Cells were cultured for 2, 4, 8 and 16 days, starting with 50000 cells in 4 ml of medium for all cell lines. TGF-β1 in supernatants was activated by adding 1 N HCL (100 μl/500 μl sample) to samples for 10 min. and subsequently neutralized by addition of 1.2 N NaOH containing 0.5 M HEPES (100 μl/500 μl sample). To correct for the TGF-β1 present in the medium, the OD_{450} values of the HCL treated medium were subtracted from the OD_{450} value of the supernatants. All samples were assayed as triplicates.

RNA extraction

Total RNA was isolated from cells that were grown to approximately 60% confluence in 250 ml culture flasks (Greiner, Frickenhausen, Germany) using TRIzol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands). The total RNA was phenol/chloroform-extracted, ethanol precipitated and cleaned with Rneasy cleanup system columns (Qiagen GmbH, Hilden, Germany).

Oligonucleotide microarray & data analysis

Affymetrics focus arrays (Santa Clara, CA), representing 8,793 human sequences from the NCBI Refseq database, were used for mRNA expression profiling according to manufacturer's instructions. Briefly, 10 μg of purified RNA was transcribed by Superscript II reverse transcriptase (Life Technologies, Grand Island, NY) using T7-(dT)24 primer containing a T7 RNA polymerase promoter. After synthesis of the second complementary DNA (cDNA) strand, this product was used in an *in vitro* transcription reaction to generate biotinylated complementary RNA (cRNA) using the Bioarray RNA transcript labeling kit (Enzo, Farmingdale, NY). Focus arrays were hybridized with 10 μg of biotinylated RNA. After hybridization, microarrays were washed and stained on an Affymetrics fluidics station and scanned with an argon ion confocal laser. Data files of all microarrays were normalized using sequence information in Robust Multi-Chip Average (GCRMA) method [24,25]. Quality assurance was performed on the arrays using Affy-Probe Level Model (AffyPLM) [26]. Microarrays for each cell line were performed in duplicate for all conditions. Normalized data were statistically analyzed for each cell line separately. A change

in gene expression during the four time points was analyzed by one-way ANOVA using R language for statistical computing. Genes that showed significant changes ($p \leq 0.01$) in expression pattern were selected for further analysis by functional grouping on gene ontology (GO) terms and by screening the public available databases (http://www.ncbi.nlm.nih.gov and http://www.ncbi.nlm.nih.gov/omim).

Quantitative real-time PCR (qRT-PCR)

2 μg of total RNA was transcribed to cDNA with reverse transcriptase of Superscript II (Life Technologies, Grand Island, NY). Amplification reactions were performed with qPCR Core kit for Sybr Green (Eurogentec Hampshire, UK) according to manufacturer's protocol. Fluorescent PCR analysis was performed using the BIO-RAD iCycler (BIO-RAD, Hercules, CA). The following PCR conditions were used: 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at the appropriate annealing temperature. Genes for normalization of expression data were selected on basis of constant high microarray gene expression data during the 4 time points. Primers were either designed with Beacon designer (BIO-RAD), retrieved from Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb) or ordered (Tebu-Bio, Heerhugowaard, The Netherlands). Expression of the genes of interest was normalized by geometric averaging of multiple internal control genes using the Genorm program [27]. Out of 5 normalization genes the best three were selected within this program; CPSF6, EEF1A1 and RPL13. Relative quantification was performed using standard curves, followed by adjustment with the normalization factor, which is calculated by the Genorm program. Primer sequences are listed in Table 1, apart from commercially available primers for CDKN1A and NFKB2.

Table 1. Primers used in qRT-PCR.

Gene	Primers
CPSF6 sense (5'-3')	AAGATTGCCTTCATGGAATTGAG
CPSF6 antisense (3'-5')	TCGTGATCTACTATGGTCCCTCTCT
EEF1A1 sense (5'-3')	CTGGCAAGGTCACCAAGTCT
EEF1A1 antisense (3'-5')	CCGTTCTTCCACCACTGATT
RPL13a sense (5'-3')	CCTGGAGGAGAAGAGGAAAGAGA
RPL13a antisense (3'-5')	TTGAGGACCTCTGTGTATTTGTCAA
ATF4 sense (5'-3')	CCAACAACAGCAAGGAGGAT
ATF4 antisense (3'-5')	GTGTCATCCAACGTGGTCAG
TNFRSF21 sense (5'-3')	CCACAGCTCAGCCAGAACAG
TNFRSF21 antisense (3'-5')	CGGTGGCACGGTCAACAT
TNFRSF12a sense (5'-3')	CCAAGCTCCTCCAACCACAA
TNFRSF12a antisense (3'-5')	TGGGGCCTAGTGTCAAGTCT
DKK1 sense (5'-3')	TCCGAGGAGAAATTGAGGAA
DKK1 antisense (3'-5')	CCTTCTTGTCCTTTGGTGTGA
GADD45B sense (5'-3')	CCAGTCCTTCTGCTGTGACAAC
GADD45B antisense (3'-5')	TCCGTGTGAGGGTTCGTGAC
TGFBI sense (5'-3')	GCCCTGAGAGACCTGCTGAAC
TGFBI antisense (3'-5')	TCGCCTTCCCGTTGATAGTGAG
PAI-1 sense (5'-3')	CCTGGGAATGACCGACATGT
PAI-1 antisense (5'-3')	TGCGGGCTGAGACTATGACA

Results

Cell Growth response of CC10B, SiHa and HeLa to TGF-β1

The degree of TGF-β1 sensitivity was determined in cervical cancer cell lines CC10B, SiHa and HeLa at day 2, 4 and 6 of cell growth after exposure to TGF-β1, in a concentration ranging from 0.001-1 ng/ml. For all cell lines 0.1 ng/ml TGF-β1 gave plateau values in cell growth inhibition (data shown for cell line CC10B, Figure 1A). TGF-β1 induced cell growth inhibition was evident from day 1 onwards for CC10B, after 2 days for SiHa and after 4 days for HeLa. After 6 days of TGF-β1 treatment, CC10B showed approximately 75% decrease in cell growth, SiHa 50% and HeLa only 25%, demonstrating that CC10B was most, SiHa intermediate and HeLa least sensitive to TGF-β1 induced growth inhibition (Figure 1B). Only HeLa and SiHa showed an EMT-like morphology upon TGF-β1 treatment (data not shown).

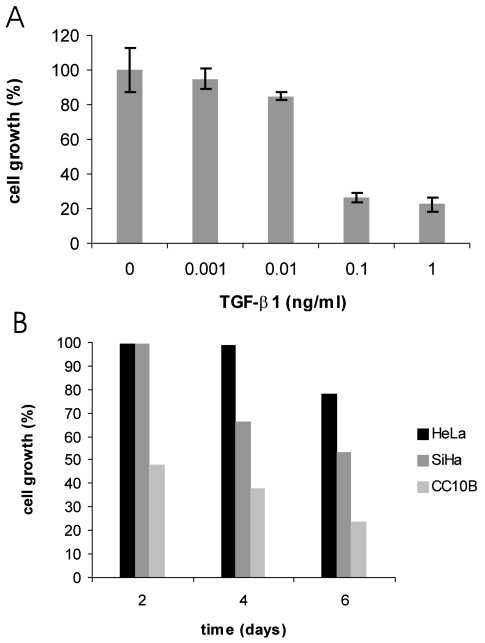


Figure 1. Effect of TGF-β1 on cell growth of cervical cancer cell lines. A) Influence of TGF-β1 concentration on cell growth inhibition of CC10B. Cell growth was determined by measuring the number of cells at day 6 using a cell counter as described in Materials and methods. Cells were stimulated with 0.001, 0.01, 0.1 or 1 ng/ml TGF-β1 in triplicate. A representative experiment is shown. B) Sensitivity for cell growth inhibition by TGF-β1 of HeLa, SiHa and CC10B. The sensitivity for cell growth inhibition by TGF-β1 was measured in HeLa, SiHa and CC10B, using a cell counter. Cells were treated with 0.1 ng/ml TGF-β1 during 6 days. For each cell line, growth inhibition by TGF-β1 was compared between cells in the absence (100%) and presence of TGF-β1 at the same time point. The assay was performed in triplicate and repeated at least 2 times.

Expression profile of CC10B, SiHa and HeLa after TGF-β1 stimulation

To determine the pathways involved in TGF-β1 induced cell growth regulation, CC10B, SiHa and HeLa were stimulated with 0.1 ng/ml TGF-β1 for 0, 6, 12 or 24 h for profiling by microarray. All samples for each cell line were performed in duplicate for statistical analysis. The reliability of the data after normalization was confirmed by AffyPLM-analysis which indicated that arrays between the four time points (0, 6, 12 and 24 h) for each cell line were comparable (data not shown). For each cell line statistically significant changes ($p \leq 0.01$) in gene expression (SSCG) were selected, calculated by one-way ANOVA analysis. The number of SSCG varied per cell line; for CC10B 253, for SiHa 140 and for HeLa 306 (Figure 2).

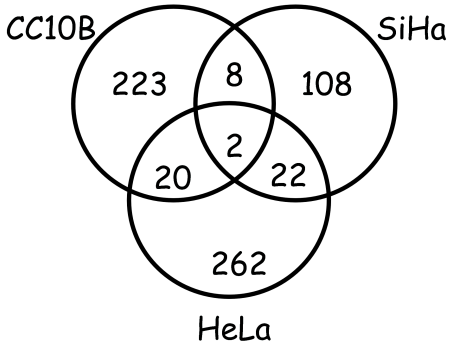


Figure 2. Number of TGF-β1 induced significant changes in gene expression (ANOVA $p \leq 0.01$). Venn diagram demonstrating SSCG for each cell line and the overlap between the cell lines. All cell lines share 2 genes, present within all 3 circles, whereas 8 genes are shared between SiHa and CC10B, 22 genes between SiHa and HeLa and 20 genes between HeLa and CC10B.

To investigate at which time point TGF-β1 stimulation resulted in the majority of changes in gene expression, the SSCG were clustered in a hierarchical tree for each cell line (data not shown). Most TGF-β1 induced gene expression changes occur after 6 h. Time points 12 and 24 h cluster separately from the other time points (0 and 6 h) indicating that in all cell lines most similarities in gene expression are shared between time point 12 and 24 h.

The percentage of SSCG in the cell lines, previously reported to be targets of TGF-β1 signaling, was determined as 4% for CC10B, 11% for SiHa and 6% for HeLa (data not shown). The majority of these genes were mainly involved in cell cycle, signal transduction, cell adhesion, and transport processes.

Next, all SSCG for each cell line were ordered according to biological processes (GO terminology). Most of the SSCG were involved in signal transduction, transport (e.g. electron, cat ion, protein, etc.) and transcription (Figure 3).

Pathways induced or altered by TGF-β1

Then, we wanted to explore which TGF-β1 effected pathways were involved in cell growth regulation in the different cell lines. Therefore, SSCG were categorized per

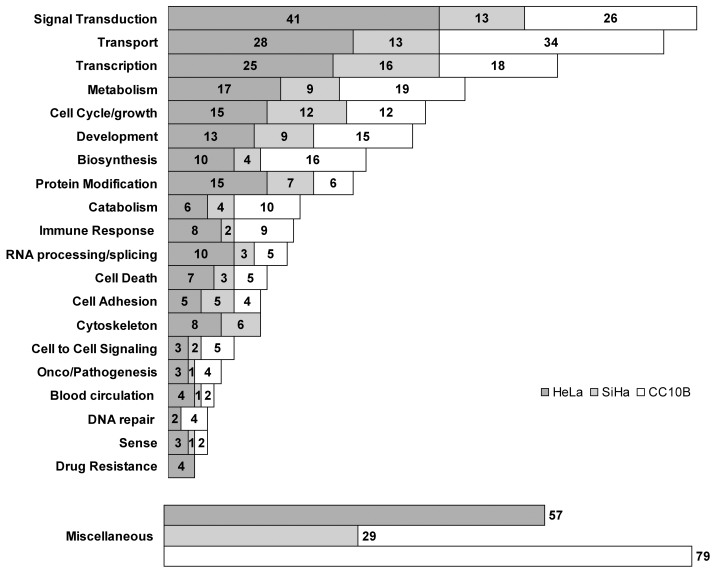


Figure 3. Biological processes demonstrating numbers of TGF-β1-induced SSCG. Bars indicate proportions of SSCG for each cell line; HeLa is depicted in dark grey, SiHa in light grey and CC10B in white. The bars are categorized by "Gene Ontology" terms.

pathway. For each cell line, growth-regulatory pathways with altered gene expressions were grouped (Figure 4A). Pathways altered in HeLa comprised MAPK, Smad, TNFα, Wnt and nuclear factor kappa B (NFKB). Pathways influenced in SiHa consisted of MAPK, Smad, TNFα, Wnt and NFKB. For CC10B the pathways influenced consisted of MAPK, Smad, TNFα, Wnt, Fibroblast Growth Factor (FGF), IFN and Rho. Subsequently, the pathways of interest between the cell lines were compared by examining the up- or downregulation of gene expression of SSCG in each particular pathway (Figure 4B). We focused on pathways affected by TGF-β1 in all cell lines: Smad, MAPK, TNFα and Wnt.

MAPK pathway

In HeLa, the cell line most resistant to TGF-β1 induced cell growth inhibition, MEF2C, a target gene of p38, and MKK6, an upstream kinase and activator of p38, were upregulated by TGF-β1. ATF4, a transcription factor possibly involved in MAPK signaling, was also increased by TGF-β1 in HeLa. Target genes of Smad and MAPK, in particular p38, pathways, such as ATF3, CDKN1A and GADD45B, were upregulated in the TGF-β1 semi resistant cell line SiHa. Besides the p38 pathway in HeLa and possibly in SiHa, the Ras pathway may be affected by TGF-β1 in HeLa and CC10B. In HeLa, *PLAT* and *MNK2*, target genes of Ras and Smad signaling, are upregulated. In CC10B, *PLAT* is upregulated in gene expression.

A

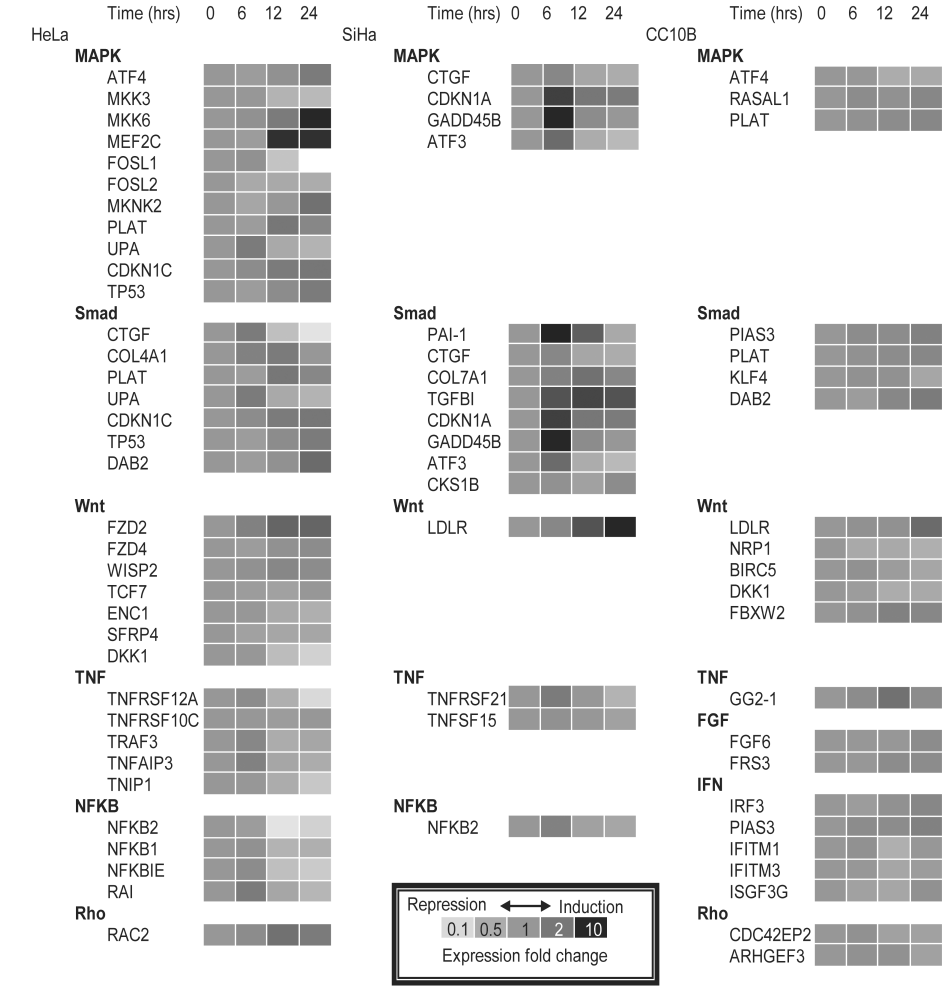
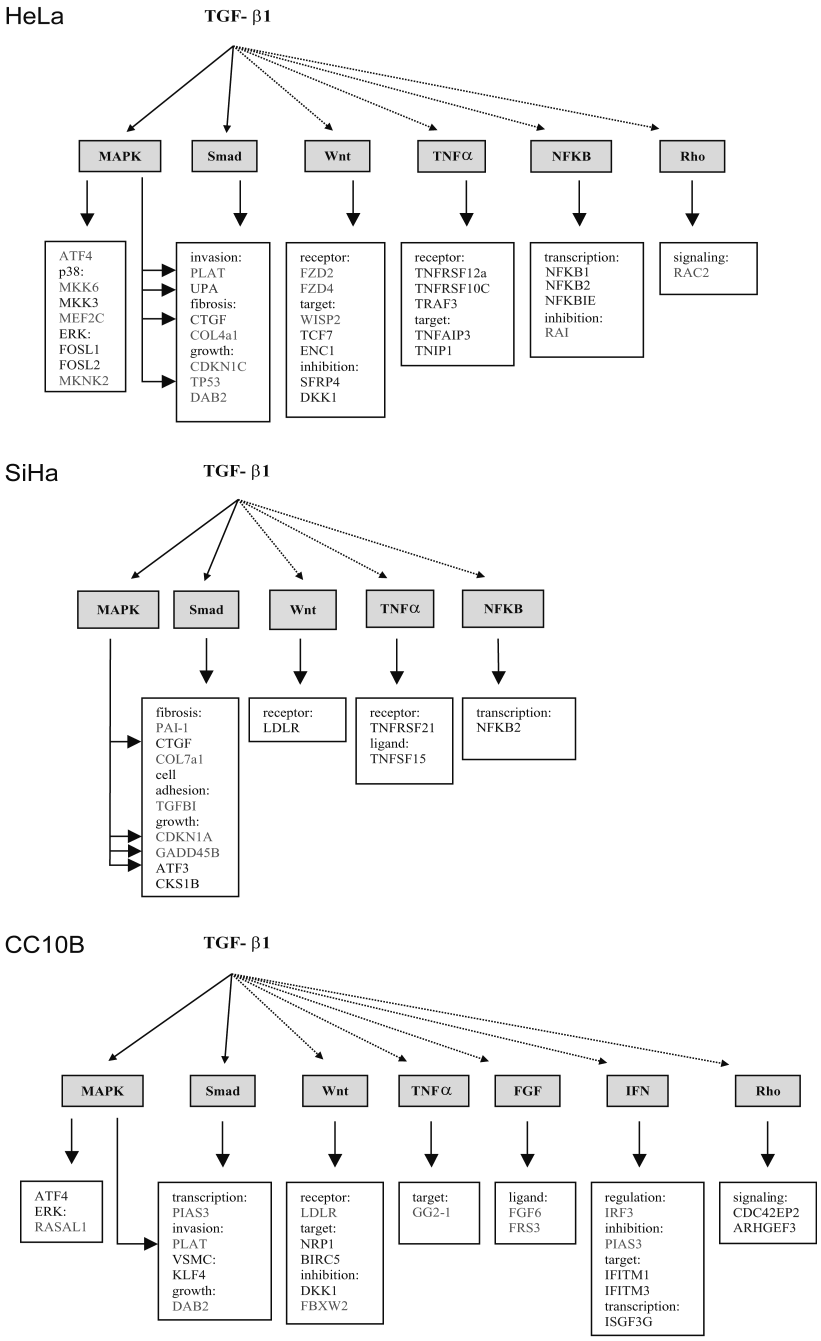


Figure 4. TGF-β1 induced alterations in gene expression. A) Expression profiles of potential TGF-β1 target genes. Changes in gene expression after TGF-β1 stimulation of HeLa (on the left), SiHa (in the middle) and CC10B (on the right), grouped by pathway. Dark colors, relative to time point 0, represent upregulation of gene expression, whereas light colors represent downregulation of SSCG. B) TGF-β1 induced changes in gene expression of pathways in HeLa, SiHa and CC10B. Pathways, shown in light gray boxes, are associated with TGF-β1 signaling and involved in regulation of cell proliferation. The boxes below the pathways show in red, genes which are upregulated in expression, in blue, genes which are downregulated and in black, genes which show evident upregulation and downregulation during the 4 time points compared to time point 0 hr. In HeLa SSCG are observed in MAPK, Smad, Wnt, TNFα, NFKB and Rho pathways, in SiHa SSCG are observed in MAPK, Smad, TNFα and NFKB pathways and in CC10B, SSCG were found in MAPK, Smad, TNFα, IFN, FGF and Rho pathways.

B


HeLa
TGF- β1
MAPK
Smad
Wnt
TNFα
NFKB
Rho
ATF4
p38:
MKK6
MKK3
MEF2C
ERK:
FOSL1
FOSL2
MKNK2
invasion:
PLAT
UPA
fibrosis:
CTGF
COL4a1
growth:
CDKN1C
TP53
DAB2
receptor:
FZD2
FZD4
target:
WISP2
TCF7
ENC1
inhibition:
SFRP4
DKK1
receptor:
TNFRSF12a
TNFRSF10C
TRAF3
target:
TNFAIP3
TNIP1
transcription:
NFKB1
NFKB2
NFKBIE
inhibition:
RAI
signaling:
RAC2
SiHa
TGF- β1
MAPK
Smad
Wnt
TNFα
NFKB
fibrosis:
PAI-1
CTGF
COL7a1
cell
adhesion:
TGFBI
growth:
CDKN1A
GADD45B
ATF3
CKS1B
receptor:
LDLR
receptor:
TNFRSF21
ligand:
TNFSF15
transcription:
NFKB2
CC10B
TGF- β1
MAPK
Smad
Wnt
TNFα
FGF
IFN
Rho
ATF4
ERK:
RASAL1
transcription:
PIAS3
invasion:
PLAT
VSMC:
KLF4
growth:
DAB2
receptor:
LDLR
target:
NRP1
BIRC5
inhibition:
DKK1
FBXW2
target:
GG2-1
ligand:
FGF6
FRS3
regulation:
IRF3
inhibition:
PIAS3
target:
IFITM1
IFITM3
transcription:
ISGF3G
signaling:
CDC42EP2
ARHGEF3

Smad pathway

In agreement with the EMT-like changes observed in HeLa and SiHa upon TGF-β1 treatment, target genes of Smad signaling which are associated with fibrosis, were upregulated in these cell lines. Genes involved in growth arrest or cell cycle control, known to be regulated by Smad, were upregulated in HeLa and SiHa. Also, in CC10B, genes involved in growth arrest such as *GADD45A* (data not shown), not previously shown to be mediated by Smad, were upregulated.

Wnt pathway

In HeLa, enhanced gene expression for two frizzled receptors; *FZD2* and *FZD4* and the target gene *wisp2*, known to be part of Wnt signaling, were observed. *SFRP4* and *DKK1*, inhibitors of Wnt signaling, were downregulated in HeLa. In CC10B, *DKK1* was downregulated as well. In SiHa and CC10B, *LDLR*, a putative Wnt coreceptor, was upregulated. No target genes of the Wnt pathway were upregulated in either CC10B or SiHa. In particular, HeLa showed an increase of target and receptor genes in the Wnt-signaling pathway.

TNFα pathway

In HeLa, a decrease in gene expression was observed of *TNFAIP3*, *TNIP1*, *TNFRSF12a*, *TNFRSF10C* and *TRAF3*. SiHa showed downregulation of *TNFRSF21* and *TNFSF15*, whereas CC10B showed upregulation of GG2-1, a target gene of TNFα signaling pathway. Thus, the cell lines with resistance to TGF-β1 showed down regulation of TNFα signaling components, in contrast to the cell line most sensitive to TGF-β1 which showed upregulation of a TNFα target gene.

Other pathways

Pathways that were not shared between all the cell lines were Rho, IFN, FGF and NFKB. As for the Rho family of small GTPases, *Rac2* was upregulated in HeLa, whereas *CDC42EP2* and *ARHGEF3* were downregulated in CC10B. Components of the IFN signaling pathway were downregulated in CC10B whereas FGF signaling components were upregulated in CC10B. Mediators of the NFKB pathway were downregulated in HeLa which coincides with downregulation of TNFα pathway genes. *NFKB2* was transiently upregulated in SiHa.

qRT-PCR verification of microarray data

To validate the microarray results, representative gene products of different pathways were subjected to QRT-PCR. The change, observed with this technique confirmed results obtained by the microarrays, although variations in magnitude of the change between the two technologies were observed. Twelve SSCG verified in the cell lines exhibited TGF-β1-induced changes in gene expression that correlated with those observed in the microarray experiments (Figure 5). The respective genes were; *DKK1* and *ATF4* for HeLa and CC10B, *NFKB2* for HeLa and SiHa, *TNFRSF12a* for HeLa, *TNFRSF21*, *GADD45B*, *TGFBI*, *PAI-1* and *CDKN1A* for SiHa.

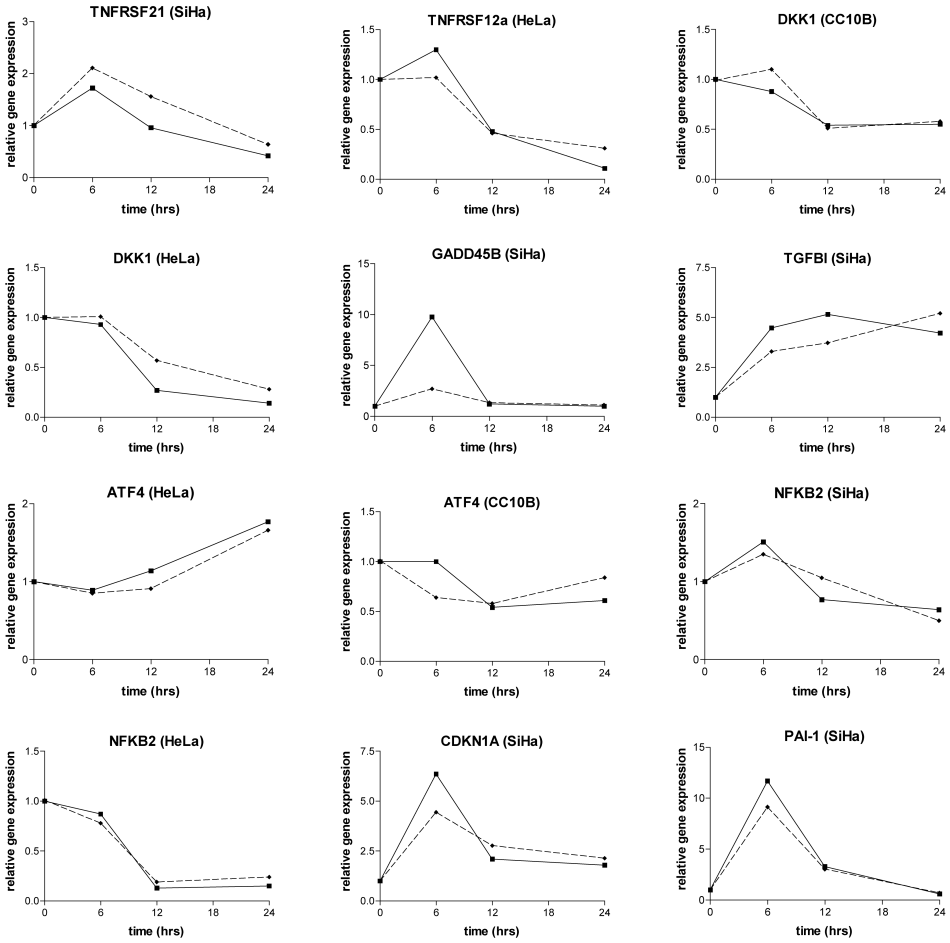


Figure 5. Correlation of expression profile between microarray and QRT-PCR. The relative expression levels of SSCG; DKK1, ATF4, TNFRSF12a, TNFRSF21, GADD45B, TGFBI, NFKB2, PAI-1 and CDKN1A were measured with microarray and QRT-PCR at 0, 6, 12 and 24 h of TGF-β1 stimulation. Data are normalized to internal control genes (see Materials and methods). The dotted line represents gene expression analyzed by QRT-PCR, the straight line represents gene expression generated by microarray.

TGF-β1 production and TGF-β1 growth inhibition

Finally, we investigated the association between decreased sensitivity to TGF-β1 mediated cell growth inhibition of the cell lines and increased TGF-β1 production. TGF-β1 production was measured in the supernatants of CC10B, SiHa and HeLa cells after 2, 4, 8 and 16 days of cell culture. Only HeLa and SiHa excreted TGF-β1. HeLa, which was most resistant to TGF-β1 induced growth inhibition, showed the highest production of TGF-β1 (Figure 6A). Subsequently, the effect of produced TGF-β1 on cell growth was determined. For this purpose SiHa cells were cultured with anti-TGF-β. Anti-TGF-β did not alter cell growth kinetics, suggesting that the produced TGF-β1 is latent TGF-β1 and does not affect cell growth (Figure 6B).

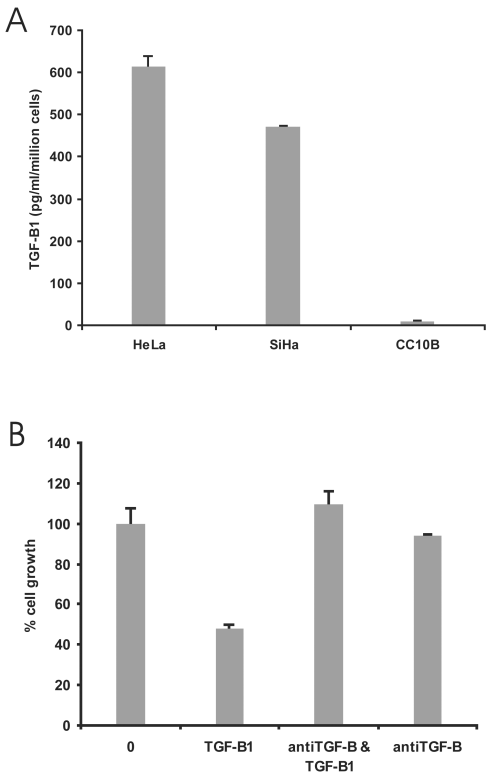


Figure 6. TGF-β1 production of the cell lines. A) The amount of TGF-β1 production, measured by ELISA, in supernatants of HeLa, SiHa and CC10B after 8 days of cell culture (measured as described in Materials and methods). To correct for the TGF-β1 present in the medium, the OD450 values of the HCL treated medium were subtracted from the OD450 value of the supernatants. B) The effect of anti-TGF-β on cell growth of SiHa. Cell growth, measured in triplicate by cell counts of SiHa, after 6 days without anti-TGF-β, with anti-TGF-β (10 μg/ml), with TGF-β1 (0.1 ng/ml) and with both TGF-β1 and anti-TGF-β. Cell number after 6 days without TGF-β1 is shown as 100%.

Discussion

The mechanisms by which cancer cells loose their TGF-β1 induced growth inhibition are not fully understood. Defects in TGF-β1 signal transduction components, thereby contributing to loss of Smad signaling, does appear in some tumor types, but does not explain the loss for growth inhibition by TGF-β1 in most cancers, including cervical cancer [28]. TGF-β1 can activate signaling pathways involved in growth control independently of Smads [15,29-31]. The role of these non-Smad signaling cascades remains to be better characterized. By means of gene expression profiling, we have investigated differences in TGF-β1 induced response using cervical cancer cell lines with different TGF-β1 sensitivity.

In all cell lines many SSCG participated in signal transduction and transcription processes. Substantial parts of SSCG were genes involved in Smad, MAPK, TNFα

and Wnt pathways. These pathways, involved in cell growth and maintenance processes are associated with TGF-β1 signaling.

Smad signaling is the main route through which TGF-β1 transduces its effects on cell cycle control. There is evident upregulation of genes which act via Smad activation and are involved in cell cycle control such as *CDKN1A*, *GADD45B* and *ATF3* [32,33] in SiHa and *CDKN1C* and *TP53* in HeLa. In CC10B, *GADD45A* [34], and *TP73L* (data not shown), negative regulators of cell cycle, were upregulated although these have not been described before as targets of Smad signaling. Besides genes involved in cell cycle regulation or apoptosis in HeLa, genes involved in cell division were upregulated namely *cyclin B2* and *G2* (data not shown). Thus, in all cell lines, genes which negatively regulate cell growth were induced, but only in HeLa significant upregulation of positive regulators of cell growth were observed, which may explain the resistance to TGF-β1 induced cell growth inhibition.

MAPK can be activated by TGF-β1 via TGF-β1 activating kinase 1 (TAK1) [35,36], which is a potent activator of the Stress activated protein kinases (SAPK/JNK) [37] and the p38 pathway [37-39]. In HeLa, increase in gene expression of *MKK6*, the main MAPK that activates p38, and *MEF2C*, a p38 downstream transcription factor [40], was observed. In a recent report, enhancement of a proliferative pathway in HeLa by TGF-β1 was shown involving the activation of MAPKs and probably not involving Smad activation [41]. They reported that in SiHa, a growth inhibitory pathway involving activation of Smads and a low level of MAPK activation was observed. In agreement with these results, we found more prominent upregulation of specific MAPK targets and signaling molecules in HeLa from our microarray data. However, Maliekal *et al.* showed extracellular response kinase (ERK) and JNK activation in HeLa, whereas, our microarray data supported p38 activation and possibly ERK activation in HeLa. Our results and these observations, showing a strong TGF-β1-dependent activation of MAPK and insufficient Smad activation by TGF-β1 in HeLa, suggest that this activation pattern may hamper the growth inhibitory effect of TGF-β1.

In HeLa we observed downregulation of TNFα receptors, signaling molecules and target genes of which *TRAF3* [42,43] and *TNFRSF12a* [44] have been associated with apoptotic processes or inhibition of cell growth. In SiHa, downregulation of *TNFSF15* [45] and *TNFRSF21* [46], genes involved in cell death and suppression of cell growth, was observed. In the TGF-β1 sensitive cell line CC10B no downregulation of TNFα signaling genes was observed. In contrast, upregulation of a TNFα target gene, *GG2-1* [47] was observed. In view of the TGF-β1 resistance of HeLa and SiHa, downregulation of receptors or signaling components involved in death signaling could be a way to impede TGF-β1 induced apoptosis or cell growth inhibition since TGF-β1 and TNFα can cooperate in the apoptotic process.

Aberrant Wnt signaling, often leading to activation of the pathway, occurs in many types of cancer including cervical cancer [48,49]. In HeLa upregulation of *FZD2* and *FZD4* was observed. These are receptors of the Frizzled family which transduce signals culminating in intracellular calcium release [50], activation of calmodulin-dependent kinase II [51], PKC [52] and MAPK pathway without activating the canonical β-catenin pathway. Upregulation of FZD2 [53,54] and FZD4 [55] in cancer

has been shown and suggests a role for Wnt signaling via FZD receptors in tumorigenesis. Downregulation of potent Wnt inhibitory factors such as *DKK1* and *SFRP4*, as observed in HeLa, may enhance Wnt signaling. *Wisp2*, a Wnt-1 target gene of the canonical signaling pathway, probably involved in transformation processes [56] is upregulated in HeLa. Since upregulation of Wnt signaling components and downregulation of Wnt inhibitory factors were observed, the Wnt pathway may be primed and play a role in reduced cell growth inhibition in Hela by TGF-β1.

With regard to oncogenes, in HeLa more than twice as many oncogenes were upregulated than in CC10B and SiHa (data not shown). Increased expression of proto-oncogenes may also provide a mechanism by which tumor cells show diminished responsiveness to TGF-β1 and can escape it's tumor suppressor effects. Cells undergoing EMT have a more migratory and invasive phenotype, which is a characteristic phenotype of cancer cells resistant to TGF-β1 induced cell death. In agreement with the observation of an EMT-like appearance of SiHa [5] and HeLa in response to TGF-β1, these cells are less sensitive to the antiproliferative effect of TGF-β1, which may associate with increased production of TGF-β1. TGF-β1 produced by SiHa was present in a latent form and did not inhibit cell growth since anti-TGF-β did not affect cell expansion. TGF-β1 excretion by tumor cells more likely contributes to paracrine stimulation of tumor development.

In our study novel target genes involved in TNFα, MAPK and Wnt pathways in response to TGF-β1 were found. Substantial differences in the Smad, TNFα, MAPK and Wnt pathway between TGF-β1 sensitive and insensitive cell lines were observed. Since these pathways are involved in integral processes of cell proliferation and cell death, these pathways may play a role in TGF-β1 induced cell growth inhibition.

Acknowledgements

We thank N. ter Haar for technical assistance with the ELISA and QRT-PCR and H. Baelde, M. Lombaerts, T. van Wezel and A. Cleton for advice in microarray and QRT-PCR techniques. The study was supported by the Dutch Cancer Society, grant no. RUL2001-2465. This study was done within the Centre for Medical Systems Biology (CMSB), a centre of excellence supported by the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research(NWO).

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5

Expression of Smad2 and Smad4 in cervical cancer: absent nuclear Smad4 expression correlates with poor survival

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Modern Pathology. 2008; 21(7): 866-875

Alteration in TGF-β signaling, due to a decrease in Smad2 and especially Smad4 expression have primarily been reported in pancreatic and colorectal cancers, although loss of the chromosomal region 18q21.1, containing the loci of *Smad2* and *Smad4*, is among the most frequent molecular alteration in cervical cancer. The aim of our study was to investigate whether decreased Smad2 and Smad4 protein expression in primary cervical cancers is associated with molecular alterations at 18q21.1, mutations in *Smad2* and *Smad4* functional domains or hypermethylation and to asses the biological relevance of decreased Smad2 and Smad4 expression. Subsequently, Smad2, Smad4 and p21 protein expression was determined by immunohistochemistry in 117 primary cervical carcinomas, assembled in a tissue-array. Smad signaling was shown to be associated with protein expression. All the tumors expressed Smad2 or Smad4. Weak cytoplasmic Smad2 or weak cytoplasmic Smad4 expression could not be attributed to loss of heterozygosity at 18q21.1. Despite weak/moderate Smad2 expression and absent nuclear Smad4 expression, the coding regions of the functional MH1 and MH2 domains of *Smad2* and *Smad4* were unchanged, as assed by sequence analysis. The *Smad4* promoter region was unmethylated in tumor samples with weak/moderate cytoplasmic Smad4 expression. Remarkably, both weak cytoplasmic Smad4 expression and absent nuclear Smad4 expression significantly correlated with poor disease free ($p = 0.003$ and $p = 0.003$, respectively) and overall 5-year survival ($p = 0.003$ and $p = 0.010$, respectively). Our findings support the hypothesis that Smad4 is a target molecule for functional inactivation in cervical cancer.

Introduction

Cervical cancer is caused by persistent infection with high risk human papillomavirus (HPV). In addition to infection with HPV, molecular alterations of tumor suppressor genes and/or oncogenes are imperative for the development of cervical cancer. A characteristic molecular alteration associated with malignant progression is the loss of responsiveness to transforming growth factor β (TGF-β1) induced cell growth inhibition [1,2]. TGF-β1 acts as a double edged sword, early in tumorigenesis it operates as a tumor suppressor, by inhibiting epithelial cell proliferation, later in tumorigenesis, it operates as an angiogenic factor and it suppresses an efficient anti-tumor immune response. Loss of TGF-β1 responsiveness is thought to be due to alterations in the TGF-β1 signal transduction route [3]. A mechanism, by which loss of the tumor suppressor function of TGF-β can occur, is through inactivation of *Smad* genes. As transcription factors, Smads are important for inducing the expression of cell cycle inhibitors, such as p15, p21 and p27, thereby facilitating TGF-β-mediated inhibition of cell growth [4].

Loss of heterozygosity (LOH) at 18q, the locus of *Smad2* and *Smad4*, is a common mechanism for inactivation of *Smad4* in pancreatic tumors [5]. In cervical carcinomas, LOH at 18q was observed in 10 to 37% of cases and has been associated with poor prognosis [6,7]. In addition to LOH, mutations in *Smad4* in pancreatic cancer and colorectal cancer, resulting in loss of expression or function, have been frequently reported [5,8]. Also, *Smad2* mutations have been found in colorectal cancer [9]. For cervical cancer, *Smad4* mutations were reported in cervical cancer cell lines [10] and a *Smad2* mutation in a cervical tumor has been described [11]. In addition to LOH and gene mutations, other mechanisms such as CpG methylation of the Smad promoter may play a role in down regulation of Smad expression. Recently, *Smad4* promoter methylation was reported in Barret's oesophagus and gastric carcinoma as a mechanism for decreased Smad4 expression [12,13].

The aim of our study was to investigate whether decreased Smad2 and Smad4 protein expression in primary cervical carcinomas was associated with molecular alterations at 18q21.1, mutations in *Smad2* and *Smad4* functional domains or hypermethylation and to asses the biological relevance of decreased Smad2 and Smad4 expression.

For this purpose we have determined protein expression of Smad2, P-Smad2, Smad4 and p21, by immunohistochemistry on a tissue array of 117 primary cervical cancers. Subsequently, the association between Smad2 and Smad4 protein expression and molecular alterations was investigated by determining LOH status at 18q21.1 (*Smad2* and *Smad4* loci), the occurrence of mutations in the functional domains of *Smad2* and *Smad4* and CpG methylation of the *Smad4* promoter. Furthermore, to asses the biological relevance of our findings, we have correlated the experimental results with clinico-pathological parameters.

Materials and Methods

Patient material

From 117 patients with cervical squamous carcinoma who underwent a radical hysterectomy combined with pelvic lymph adenectomy between 1985 and 2000, formalin fixed paraffin-embedded tissue blocks were retrieved from the Department of Pathology, Leiden University Medical Center. None of the patients had received radio-or chemotherapy prior to surgery. The 117 primary cervical carcinomas consisted of 90 squamous cell carcinomas, 19 adenocarcinomas and 8 adenosquamous carcinomas. The use of clinical material was approved by the institutional review board according to the Dutch federation of Medical Research Associations. The clinical records of the women, all treated by the same three gynecologic oncologists of the Department of Gynecology, Leiden University Medical Centre, with a radical hysterectomy type III for carcinoma of the uterine cervix between 1985 and 2000, were reviewed. The slides of all tumors were examined, using conventional histological sections stained with hematoxylin and eosin, by a trained pathologist.

Immunohistochemistry

Immunohistochemistry on a tissue-array was performed on 4 µm sections using aminopropylethoxysilane (APES)-coated slides. A representative tumor area was selected by an experienced pathologist. Subsequently, three cores of each tumor were included in the tissue array. Standard immunohistochemical staining was performed as previously described [14]. In case of immunohistochemistry with the Smad2, P-Smad2 and Smad4 antibodies, antigen retrieval was performed with 0.01 M citrate buffer. mAb directed against Smad4 (clone B8; dilution 1:200, Santa Cruz, CA, USA) [15], P-Smad2 (clone 138D4, dilution 1:50, Cell Signaling Technology, Beverly, CA) [16] and p21 (clone EA10, dilution 1:200, Calbiochem, CA, USA) [17] and a polyclonal antibody against Smad2 (dilution 1:200, Santa Cruz, CA, USA) [18] were used. The cellular infiltrate provided an internal positive control (for Smad2 and Smad4). IHC was scored as previously described [19]. Intensity of cytoplasmic staining was scored as weak, moderate or strong at low magnification (100x). In case of nuclear staining the percentage of positive tumor cells was determined and divided into three groups: nuclear staining in 0% of tumor cells (absent), nuclear staining in > 0% of tumor cells and ≤ 50% of tumor cells (low) or nuclear staining in > 50% of tumor cells (high). Expression was scored by two independent researchers without knowing the clinical outcome of patients.

Genomic DNA isolation from flow-sorted tumor cell subpopulations

Formalin-fixed, paraffin-embedded cervical carcinomas were trimmed if necessary to remove normal epithelium and cervical intraepithelial neoplasia. The remaining tumor tissue was cut into 50 µM sections, dewaxed and pretreated with 10 mM sodium citrate buffer (pH 6,4) as antigen retrieval. Dissociation of tissue was performed in a mixture of 0,1% collagenase I-a and 0,1% dispase in RPMI medium without FCS and antibiotics for 30-120 minutes at 37°C [20]. The cells were stained

for keratin, vimentin and DNA as previously described [20]. Anti-vimentin antibodies applied were 3B4 (2 μg/ml; DAKOCytomation, Glostrup, Denmark) and V9-2b (supernatant diluted 1:5). Monoclonal antibodies used against keratin include MNF116 (2 μg/ml; DAKOCytomation, Glostrup, Denmark) and Pan-keratin AE1/AE3 (5 mg/ml; Chemicon International Inc, Temecula, CA, USA). The vimentin-negative keratin-positive (V-K+) fraction, which represents the epithelial cell subpopulation, and vimentin-positive keratin-negative (V+K-) fraction, consisting of normal cells, were flow sorted using a FACS Vantage (BD Biosiences, San Jose, CA). The number of sorted cells varied from 50.000 to 1.000.000 cells between samples. DNA was extracted as previously described [21].

Microsatellite analysis

Seven Hex-labeled primer pairs of microsatellite markers comprising D18s877, D18s65, D18s460, D18s1137, D18s1118, D18s1109 and D18s844 (Genome Database, http://www.gdb.org) at chromosome 18q, were used. The nearest marker for Smad2 is D18S1137 at 0.2 MB distance and for Smad4, D18S1118 at 2.6 MB. DNA, isolated from sorted tumor cell subpopulations, was used as source material [20]. PCR, electrophoresis and analysis was performed as described previously [22]. A reduction of 50% of one allele, equating to a ratio of ≥ 2 or ≤ 0.5 was assigned as LOH. A reduction of 25% or more (1.5-2 and 0.75-0.5) of one allele was assigned as allelic imbalance.

Mutation analysis

Primers designed for amplification of the complete coding region of MH1 and MH2 domains of *Smad2* and *Smad4*, applicable for paraffin material as template, are listed in supplementary Table 1 and Table 2. The following PCR conditions were used: 10 min at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 53°C and 30 seconds extension at 72°C. Sequencing was performed by the Leiden Genome Technology Center (Leiden, The Netherlands). The obtained sequences were compared with DNA sequences of autologous normal material and with the corresponding sequences from the Genome Database (http:/www.gdb.org).

Methylation assay

For the detection of CpG methylation in cell cultures, cervical cancer cell lines and sorted tumor cell subpopulations, the bisulphite method was applied for DNA methylation analysis using the EZ DNA Methylation Kit (ZYMO Research, Orange, USA) according to the manufacturer's protocol. Prior to conversion with the bisulphite method, DNA from sorted samples was purified and concentrated with Wizard DNA clean-up (Promega, Madison, WI). DNA input for the conversion reaction was 500 ng/sample. Designed primers for PCR and sequencing of bisulphite-converted DNA, encompassing *Smad4* promoter and exon 1 region, were as follows:
5'-GGTTGGGTTAATAGTTATTTATA (FWD) and CTCTAAATCTAAACCTAAACCTA (REV), 5'-TAGGTTTAGGTTTAGATTTAGAG (FWD) and AATTTAACCCTTTTCCCCCC (REV), 5'-GGGGGAAAAGGGTTAAATT (FWD) and CTAAACACCTAACTCCCCTC (REV).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL). Data were processed using the Chi-square test. Kaplan-Meier survival curves were generated to assess differences in disease-free survival (defined as the time in months from surgery to relapse of the disease) or cumulative overall survival (defined as time in months from surgery to death due to cervical cancer). A Cox regression was used for univariate and multivariate survival analysis. $P \leq 0.050$ was considered statistically significant.

Results

Smad2, P-Smad2, Smad4 and p21 protein expression in primary cervical carcinomas

Protein expression of Smad2, P-Smad2, Smad4, and p21 was evaluated by immunohistochemistry in a tissue array containing 117 primary cervical carcinomas. Because of its nuclear localization p21 was chosen as a target product for TGF-β signaling. All tumors showed a homogeneous cytoplasmic staining pattern for Smad2 and Smad4 that varied in intensity (Figure 1 and Table 1). For analysis of the cytoplasmic staining results the three categories from Table 1 were combined into two categories (weak versus moderate/strong). Weak cytoplasmic Smad2 staining intensity correlated significantly with weak cytoplasmic Smad4 staining intensity (p = 0.050). All tumors showed nuclear staining for P-Smad2 (Table 1). Fifteen out of 111 tumors lacked nuclear staining for Smad4 (14%) and fifteen out of 114 tumors lacked nuclear staining for p21 (13%). The P-Smad2 consisted of two groups, a group with less than 50% positive tumor cells (low) and a group with more than 50% positive tumor cells (high). To analyze the results of the nuclear staining pattern,

Table 1. Differential expression of Smad2, P-Smad2, Smad4 and p21 proteins in cervical cancers.

	Percentage positive tumor cells		
Nuclear	0% (absent)	1-50% (low)	51-100% (high)
	N (%)	N (%)	N (%)
P-Smad2	0 (0)	23 (20)	93 (80)
Smad4	15 (13)	71 (64)	25 (23)
p21	15 (15)	94 (90)	5 (5)

	Intensity*		
Cytoplasmic	Weak	Moderate	Strong
	N (%)	N (%)	N (%)
Smad2	37 (33)	58(52)	17(15)
Smad4	40 (36)	53(48)	18(16)

Expression of Smad2, P-Smad2, Smad4 and p21 was measured by immunohistochemistry as described in Materials and Methods. N, number of tumors. (%), Percentage of tumors displaying either a nuclear or a cytoplasmic staining pattern for P-Smad2, Smad2, Smad4 or p21. *, Staining intensity (weak, moderate or strong).

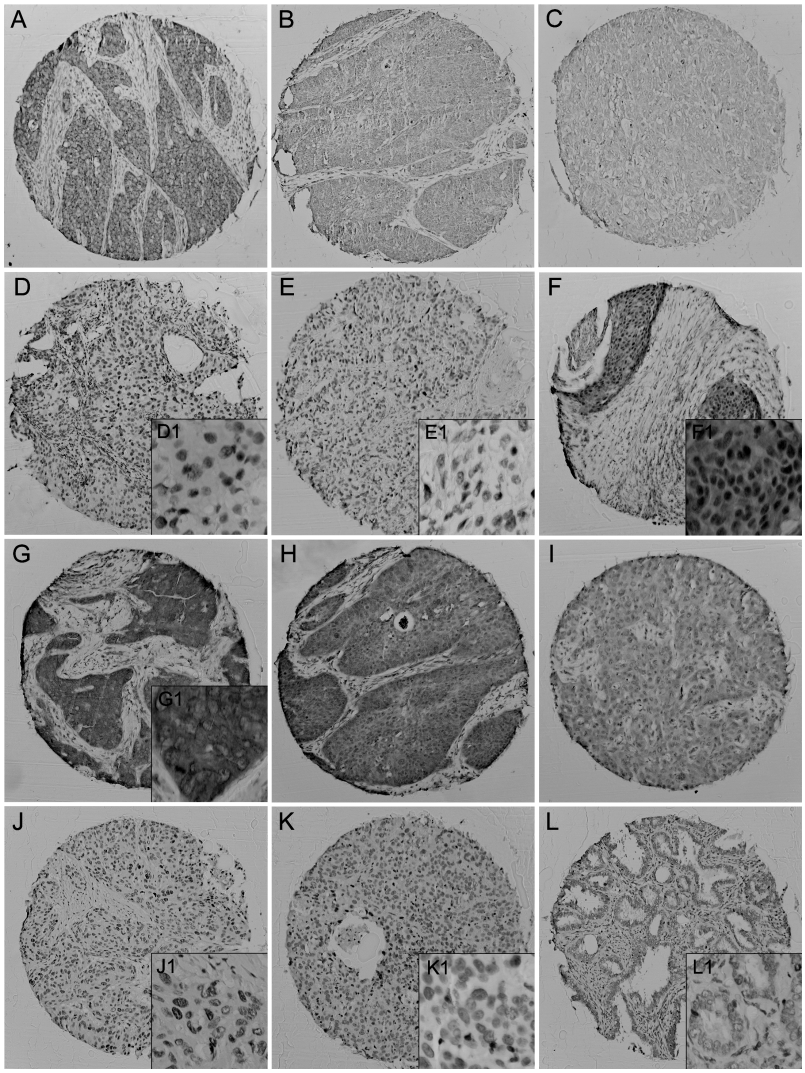


Figure 1. Expression of Smad2, Smad2-P, Smad4 and p21 proteins in primary cervical carcinomas. A tissue-microarray of 117 cervical tumors was stained using immunohistochemistry as described in Materials and Methods (125x magnification). A: Smad2, strong cytoplasmic staining, B: Smad2, moderate cytoplasmic staining, C: Smad2, weak cytoplasmic staining, D: positive nuclear staining of Smad2-P in > 50% of tumor cells, D1: detail of D (400x magnification), E: Smad2-P nuclear staining in ≤ 50% of tumor cells, E1: detail of E (400x magnification), F: Smad4, positive nuclear staining in > 50% of cells, F1: detail of F (400x magnification), G: Smad4, strong cytoplasmic intensity and absent nuclear staining, G1: detail of G (400x magnification), H: Smad4, moderate cytoplasmic intensity, I: Smad4, weak cytoplasmic intensity, J: p21 nuclear staining in > 50% of tumor cells, J1: detail of J (400x magnification), K: p21 nuclear staining in > 0% and ≤ 50% of tumor cells, K1: detail of K (400x magnification), L: absent p21 nuclear staining and L1: detail of L (400x magnification).

nuclear Smad4 and p21 were divided in a group without nuclear staining (absent) and a group with nuclear staining. A P-Smad2 nuclear staining pattern (1-50%) showed a significant correlation with absence of p21 staining ($p = 0.011$). Although not significant, a trend was observed for the association between absence of nuclear Smad4 and absence of p21 ($p = 0.051$). In addition, a significant association between weak cytoplasmic Smad4 staining intensity and absent Smad4 nuclear staining was observed ($p = 0.001$).

LOH of the Smad2 and Smad4 loci at 18q21.1

To establish the basis for weak Smad2 and weak Smad4 protein expression, we have investigated whether this was due to haplo-insufficiency of the *Smad2* or *Smad4* genes caused by LOH. For LOH analysis, DNA was used from a flow sorted tumor cell subpopulation (keratin positive, vimentin negative; K+V-) to prevent contamination by either stromal or inflammatory cells. LOH at 18q21.1 was determined in 46 tumors with low, moderate and strong expression of either Smad2 or cytoplasmic Smad4 (Figure 2B). High allelic imbalance ratios were frequently observed, often ranging from 2-10, representing complete LOH. From the 46 cases, 19 cases (41%) showed loss at markers flanking the *Smad2* and *Smad4* loci (allelic imbalance = 1.5). No significant association between Smad2 or Smad4 protein expression and LOH at 18q21.1 was apparent.

Mutation analysis of the functional MH1 and MH2 domains of Smad2 and Smad4

Subsequently, we investigated whether mutations in the function associated MH1 and MH2 domains of *Smad2* or *Smad4* were the underlying cause for their reduced expression. For this purpose, 5 tumors with a weak and 5 tumors with a moderate cytoplasmic Smad2 staining pattern were selected (all the tumors selected displayed less than 50% of P-Smad2 positive tumor cells). For Smad4, 8 tumors with weak and 2 tumors with a moderate cytoplasmic Smad4 staining pattern were selected (without nuclear Smad4 staining). PCR-amplification and sequence analysis of DNA from the flow sorted tumor cell subpopulation (V-K+) as well as DNA from the flow sorted stromal cell/inflammatory cell subpopulation (V+K-) as a control, were performed. No mutations in *Smad2* and *Smad4* MH1 and MH2 domains were observed (data not shown).

Methylation of the promoter region and exon 1 of Smad4

Because of lack of significant association between Smad2 or Smad4 expression and LOH at 18q21.1 and the absence of mutations in *Smad2* and *Smad4* MH1 and MH2 domains, we have investigated whether methylation of the promoter region could explain the reduced protein expression. We focused on *Smad4* promoter methylation since *Smad4* promoter methylation has been described as a tumor suppressor mechanism previously [12,13]. Four cervical cancer cell lines (HeLa, CaSki, CC-8 and CC-11) and four normal primary cervical epithelial cell cultures, both expressing the complete coding region of *Smad4* (as determined by RT-PCR), were sequenced after bisulphite conversion to determine the CpG methylation status.

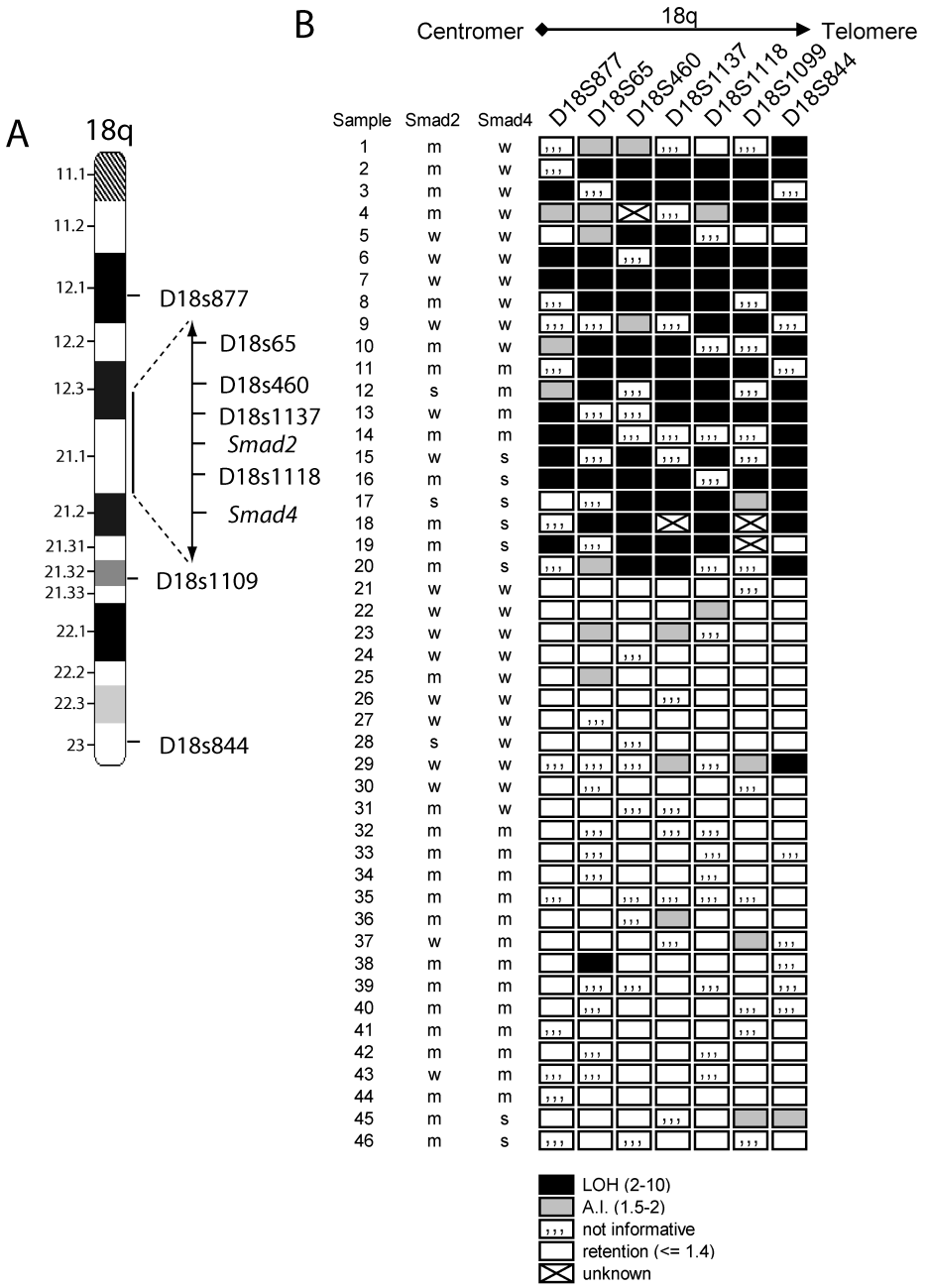


Figure 2. LOH at 18q21.1 in primary cervical carcinomas. (A) Microsatellite markers surrounding Smad2 and Smad4 genes at chromosome arm 18q. (B) LOH at 18q of 46 samples was measured using 7 microsatellite markers as described in Materials and Methods. For each tumor Smad2 and cytoplasmic Smad4 staining intensity is depicted as w: weak, m: moderate and s: strong.

Also DNA from 5 flow-sorted tumor cell populations with weak and 5 flow-sorted tumor cell populations with moderate Smad4 cytoplasmic staining were analyzed for CpG methylation status. The region up to 300 bp upstream from the transcription start point, containing dense CpG islands, and 125 bp downstream of the transcription start, which includes exon 1, was sequenced (supplementary Figure 1). None of the CpG sites investigated was methylated (data not shown).

Association between Smad2, Smad4 and p21 expression with clinico-pathological parameters

Despite the unknown nature of the decreased Smad2 and cytoplasmic Smad4 expression, we have investigated whether the expression pattern of Smad2, P-Smad2, cytoplasmic Smad4, nuclear Smad4 and p21 correlated with clinico-pathological parameters (Table 2).

Table 2. Significant association between weak cytoplasmic Smad4, absent nuclear Smad4 expression and clinico-pathological parameters.

		Smad4					
Clinico-pathological characteristics		Cytoplasmic			Nuclear		
		Weak	Mod./Strong		Absent	Present	
		N		P	N		P
Lymph node		108			109		
	Positive	17 (61)	11 (39)	**0,002**	6 (21)	22 (79)	0,115
	Negative	22 (28)	58 (72)		8 (10)	73 (90)	
Recurrent disease		100			100		
	Yes	15 (40)	23 (60)	**0,009**	6 (24)	19 (76)	0,059
	No	23 (31)	52 (69)		7 (9)	68 (91)	
Tumor size		103			104		
	< 40 mm	18 (29)	45 (71)	0,051	5 (8)	58 (92)	**0,02**
	≥ 40 mm	19 (48)	21 (52)		10 (24)	31 (76)	
Depth of infiltration		109			110		
	< 15 mm	19 (30)	45 (70)	0,07	5 (8)	59 (92)	**0,036**
	≥ 15 mm	21 (47)	24 (53)		10 (22)	36 (78)	
HPV type		80			80		
	16	20 (36)	36 (64)	0,614	5 (9)	51 (91)	**0,02**
	18	10 (42)	14 (58)		7 (29)	17 (71)	

N, number of tumors displaying a either a cytoplasmic Smad4 or nuclear Smad4 staining pattern for the provided clinico-pathological parameters. Mod., moderate. *, Only data for cervical carcinoma samples positive for HPV16 and HPV 18 were included.

Weak cytoplasmic Smad4 staining intensity strongly correlated with the presence of positive lymph nodes ($p = 0.002$) and recurrent disease ($p = 0.009$). Absent nuclear Smad4 correlated with tumors ≥ 40 mm ($p = 0.020$), infiltration depth ≥ 15 mm ($p = 0.036$) and the presence of HPV18 ($p = 0.020$). Both weak Smad2 expression

and P-Smad2 (≤50% of the tumor cells) were not significantly associated with any of the clinico-pathological parameters tested.

Because of the significant correlation between a weak cytoplasmic Smad4 staining intensity and lymph node metastases and recurrent disease, we also assessed the association between the Smad protein expression patterns and disease-free and overall 5-year survival. Indeed, weak cytoplasmic Smad4 staining intensity correlated with both poor disease free survival (p = 0.003; Figure 3A) and poor overall survival (p = 0.003; Figure 3B). Also absence of nuclear Smad4 correlated with poor disease free survival (p = 0.010; Figure 3C) and poor overall survival (p = 0.003; Figure 3D).

Subsequently we used the Cox proportional hazards model to calculate the predictive value for a shorter disease-free and overall 5-year survival of each of these parameters. Both weak cytoplasmic Smad4 intensity and absent nuclear Smad4 were demonstrated to be prognostic factors for disease free and overall 5-year survival (Table 3).

Furthermore, in a multivariate Cox analysis that included the four strongest prognostic factors according to univariate Cox analysis (lymph node status, tumor size, vaso-invasion and depth of infiltration) [23], in addition to lymph node status, absent nuclear Smad4 staining was found to be the only independent prognostic factor for both disease free (hazard ratio of 2.91) and overall 5-year survival (hazard ratio of 2.74).

Table 3 Prognostic value of weak cytoplasmic Smad4 and absent nuclear Smad4.

Parameter	Limits	DFS			Overall survival		
		HR	95% CI	P	HR	95% CI	P
Univariate Cox analysis							
Lymph nodes	Positive vs. negative	7,16	3.16-16.25	**0,001**	5,69	2.53-12.82	**0,001**
Infiltration depth	<15 mm vs.≥15 mm	3,64	1.57-8.44	**0,003**	3,96	1.64-9.55	**0,002**
Tumor size	<40 mm vs. ≥40 mm	4,24	1.79-10.03	**0,001**	7,9	2.92-21.37	**0,001**
Vaso-invasion	Positive vs. negative	3,41	1.51-7.70	**0,003**	2,76	1.24-6.17	**0,013**
Smad4 cytoplasmic	Weak vs. mod./strong	3,23	1.41-7.38	**0,006**	3,45	1.44-8.23	**0,005**
Smad4 nuclear	Absent vs. present	3,15	1.25-7.96	**0,015**	3,61	1.48-8.88	**0,005**
Multivariate Cox analysis							
Lymph nodes	Positive vs. negative	4	1.40-11.45	**0,01**	4,4	1.55-12.51	**0,006**
Tumor size	<40 mm vs. ≥40 mm	2,37	0.91-6.15	0,078	5,31	1.88-14.98	**0,002**
Vaso-invasion	Positive vs. negative	1,5	0.59-3.81	0,399	0,65	0.54-3.37	0,52
Infiltration depth	<15 mm vs. ≥15 mm	1,8	0.59-5.53	0,305	4,15	0.64-5.32	0,273
Smad4 nuclear	Absent vs. present	2,91	1.08-7.89	**0,035**	2,74	1.07-6.78	**0,035**

DFS, disease free survival. HR, hazard ratio. CI, confidence interval, vs, versus.

Discussion

Loss of responsiveness to TGF-β induced cell growth inhibition during cancer development is a feature of many tumors, including cervical cancer [1]. Although a number of studies have investigated the expression status and alterations in the canonical TGF-β signaling (Smad) pathway, the role of Smad signaling in the progression of cervical cancer remains unclear [3,10,11,24]. In this study we have investigated whether decreased Smad2 and Smad4 protein expression in primary cervical cancers was associated with molecular alterations at 18q21.1, mutations in

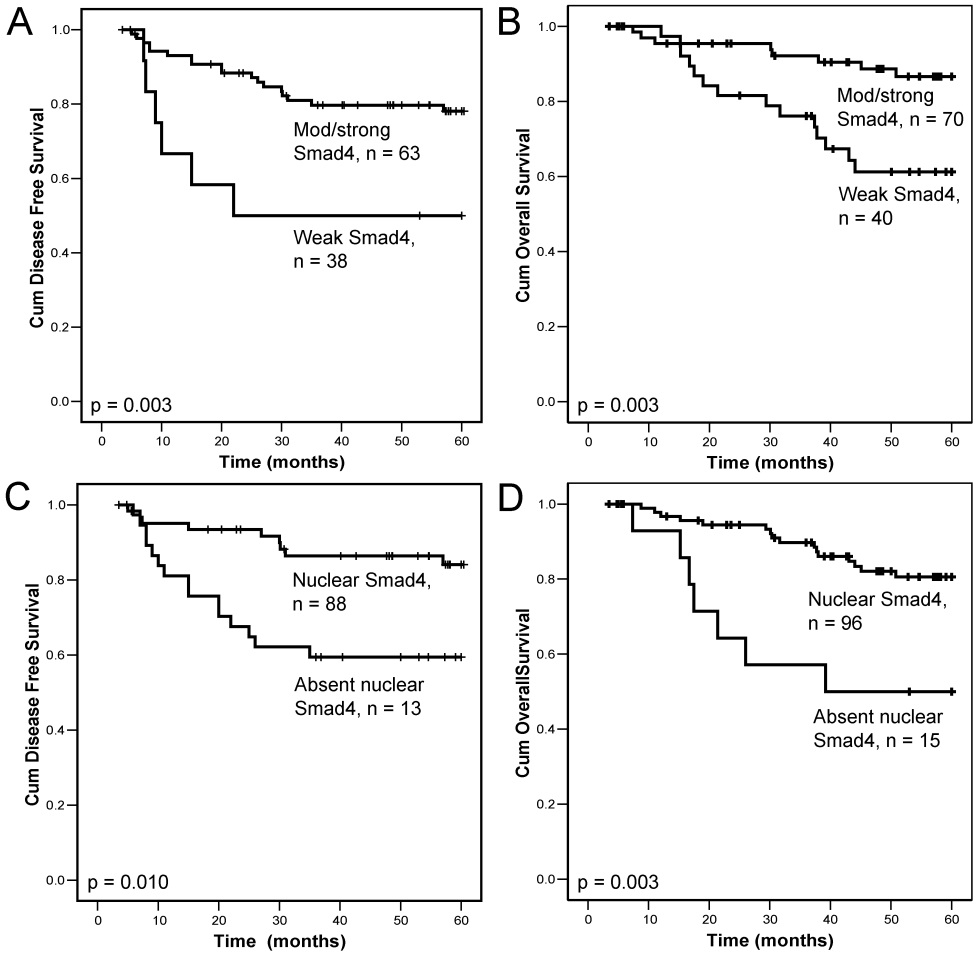


Figure 3. Association of cytoplasmic and nuclear Smad4 expression with survival in primary cervical carcinomas. Kaplan-Meier survival curves were generated to assess differences in disease-free survival (defined as the time in months from surgery to relapse of the disease) or cumulative overall survival (defined as time in months from surgery to death due to cervical cancer) as described in Materials and Methods. (A) Disease free 5-year survival in patients with weak cytoplasmic Smad4 staining intensity (n = 38) and moderate/strong intensity (n = 63) (B) Overall survival in patients with weak cytoplasmic Smad4 intensity (n = 40) and moderate/strong intensity (n = 70) in primary cervical carcinomas. (C) Disease free survival in patients with absent (n = 13) and present (n = 88) Smad4 nuclear staining. (D) Overall survival in patients with absent (n = 15) and present (n = 96) Smad4 nuclear staining.

Smad2 and *Smad4* functional domains or hypermethylation and we have assessed the biological relevance of decreased Smad2 and Smad4 expression.

Previously, Smad4 deficiency has been reported in 4 out 13 cervical cancer cell lines [10]. It should be noted that from these cell lines, one cell line was derived from a lymph node metastases (HT-3) and two other cell lines (C4-I and C4-II) were derived from the same patient. The origin of the fourth cell line was not specified. In contrast

to Baldus et al. [10], who reported a Smad4 deficiency in 10 out of 40 invasive cervical carcinomas, we did not observe tumors negative for Smad4 staining. The explanation for this difference is unclear. Baldus et al. did not report the FIGO stage of the investigated tumors, while in our study only FIGO stage IB and IIA cervical carcinomas were investigated. Conceivably differences in Smad4 expression may occur as late events during tumor progression. The expression of either P-Smad2, nuclear Smad4 or p21 in the majority of the cervical carcinomas investigated, suggests that the canonical TGF-β signal pathway is operative. However, in cases with a limited amount of cytoplasmic Smad4, the amount of Smad4 may be rate limiting.

Loss or decrease in Smad protein expression can be due to genetic alterations such as LOH and inactivating mutations [5]. We found a relatively high frequency of LOH at 18q21.1, the locus of *Smad2* and *Smad4* genes (41%) compared to other studies (10-37%) [6,7]. The close proximity of the markers to the *Smad2* and *Smad4* loci in combination with the purity of the tumor samples may explain this increased frequency. For the LOH analysis, we used DNA samples from flow-sorted cancer samples. The advantage of flow-sorting over other techniques, such as laser microdissection, is the lack of contaminating stromal cells or cells from the immune system, in the tumor sample. Since LOH at 18q21.1 did not associate with weak Smad2 or weak Smad4 expression, genetic haplo-insufficiency of Smad2 and Smad4 is not supported by our data.

Only two studies have reported on mutations in the functional domains (MH1 and MH2) of *Smad2* and *Smad4* in cervical carcinoma [10,11]. Maliekal et al. identified a missense mutation at the MH1 domain of *Smad2* in a cervical tumor and reported lack of mRNA expression of the C-terminal part of the MH2 domains in 6 tumors [11]. We did not detect mutations in *Smad2* MH1 and MH2 domains in the tumor samples, therefore it remains uncertain if cervical tumors contain inactivating mutations in the MH2 domain. Nevertheless we cannot exclude the presence of less-frequently occurring mutations outside the MH1 and MH2 domains of *Smad2* and *Smad4*.

Baldus et al. reported deletions in part of the function-associated MH1 and MH2 *Smad4* domains in 4 cervical cancer cell lines [10]. In agreement with Maliekal et al., in our study we did not observe mutations of *Smad4* MH1 and MH2 domains in cervical tumors. However, our approach does not exclude the presence of possible missense mutations. Our data suggest that that genetic inactivation of *Smad2* and *Smad4* plays a minor role in cervical cancer.

Another mechanism, by which Smad expression could be downregulated, is by promoter methylation. In our study, similar to the study of Wang et al. [13] in gastric carcinoma, we have investigated both the *Smad4* promoter region and exon 1. No hypermethylation of the *Smad4* promoter or exon 1 region was observed in normal samples, cervical cancer cell lines, and tumor samples. Also in colorectal cancers no hypermethylation of the *Smad4* was observed, whereas in Barret's esophagus, a weak correlation between mRNA expression and promoter methylation was found [12,25,26]. In gastric tumors hypermethylation was rare. In this tumor type a significant correlation was reported between hypermethylation and loss of protein expression.

Weak protein expression of Smad2 and Smad4 in cervical cancer could also be due to other regulatory processes, such as an interaction of HPV16 E7 with Smad2, Smad3 and Smad4 [27], degradation by a proteasome [13] or activation of specific ubiquitin-Smad E3 ligases [28,29].

In our series, weak cytoplasmic Smad4 staining and absence of Smad4 nuclear staining was associated with poor survival in cervical cancer patients. In a previous study, our group found an association between LOH at 18q and survival [7]. Here, we did not find this association. These different results could be explained by much closer proximity of the chosen markers to the Smad2 and Smad4 loci in the present study. In our study, weak nuclear Smad4 expression was found to be an independent prognostic factor for both disease free and overall survival. Loss of nuclear Smad4 expression in gastric cancer tumors also correlated with poor survival [13]. In pancreatic cancer, Smad4 protein expression was an independent predictor for longer survival [30]. In colorectal and breast cancer, loss of Smad4 protein expression correlated with negative outcome or presence of lymph node metastasis [31,32]. These data emphasize the importance of the integrity of the canonical TGF-β pathway in carcinogenesis. Our findings support the hypothesis that Smad4 is a target molecule for functional inactivation in cervical cancer.

Acknowledgements

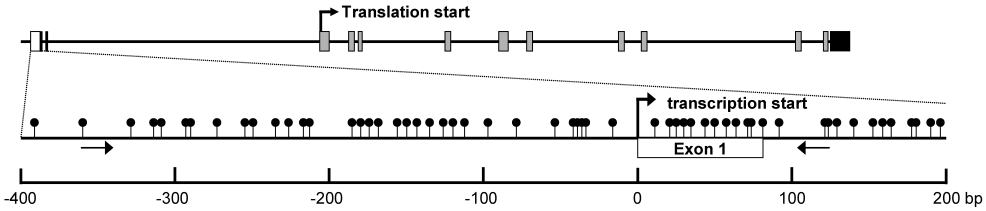
We thank P. Tholen for technical assistance with the optimization of Smad2 and Smad4 immunohistochemistry and mutation analysis. The study was supported by the Dutch Cancer Society, grant no. RUL2001-2465.

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Supplementary Figure 1. Schematic representation of the *Smad4* promoter and exon 1 region with CpG sites. The upper panel is an illustration of the complete *Smad4* gene with all exons and promoter site. Black boxes, nonprotein coding exons; grey boxes, coding exons. Below, the *Smad4* promoter and exon1 region are shown with all CpG sites covering that area. The region within the depicted arrows was analyzed by bisulphate sequencing as described in Materials and Methods. Black circles, CpG islands.

Supplementary Table 1. Primers used for amplification and sequencing of *Smad2* MH1 and MH2 domains.

Exon	Domain (fragment)	Product length (bp)	Sequence
2	MH1 (1)	167	F: 5'-AGACTGCTGGGATGGAAGAA
			R: 5'-GAAAGCCATCACCACTCAAAA
2	MH1 (2)	149	F: 5'-TGAGCTTGAGAAAGCCATCA
			R: 5'-TGTTCCTTTCAAGTTGCCTG
3	MH1 (3)	166	F: 5'-CATGCTTCCATGTTCACATCA
			R: 5'-TCTGCCCTCTGTCGAATCTT
4	MH1 (4)	191	F: 5'-CTTGATGGTCGTCTCCAGGT
			R: 5'-TCAGAGAGTTGAGACACCAGG
5	MH1 (5)	190	F: 5'-CCCTTTGCTCTTTGCCATTA
			R: 5'-AGCAGGAATTGAGCCACAGA
8	MH2 (1)	165	F: 5'-GCTTGAATTGTTCACGCATTT
			R: 5'-TGGAGAAACCTTCCATGCAT
8	MH2 (2)	127	F: 5'-GGGTTGGAGAAACCTTCCAT
			R: 5'-CGAAATGCCACGGTAGAAAT
9	MH2 (3)	153	F: 5'-CAGGAAGAGGAGTGCGCTTA
			R: 5'-TTCCACCAGGTATGGATTCAA
10	MH2 (4)	154	F: 5'-TCAGTAGATGATTGCATGCTCA
			R: 5'-GGAATTTGCTGCTCTTCTGG
10	MH2 (5)	155	F: 5'-GGAATTTGCTGCTCTTCTGG
			R: 5'-TTGGTTCTTAAATTTCCACAGTG
11	MH2 (6)	162	F: 5'-CAGAAGGCAGACGGTAACAA
			R: 5'-TCAGTGCGTTGCTCAAGC

Supplementary Table 2. Primers used for amplification and sequencing of *Smad4* MH1 and MH2 domains.

Exon	Domain (fragment)	Product length (bp)	Sequence
3	MH1 (1)	79	F: 5'-TGTGCCATAGACAAGGTGGA
			R: 5'-GGTAAAGAAGCTGAAGGAGAA
3	MH1 (2)	130	F: 5'-TTTGGTAAAGAAGCTGAAGGAGA
			R: 5'-GATGGGAGGCTTCAGGTTAGT
4	MH1 (3)	158	F: 5'-GAGTTGGTAGGATTGTGAGG
			R: 5'-AGGTGGCCTGATCTTCACAA
4	MH1 (4)	178	F: 5'-GGTGGCCTGATCTTCACAAA
			R: 5'-AACATAAAGGGAAAAGGATCTCAA
9	MH2 (1)	137	F: 5'-TGGAATTTTTGTTGTCTTTTCTTT
			R: 5'-GATTTAGTGGTGATTGAAACGC
10	MH2 (2)	155	F: 5'-CATGGGAGGATGTTCTTTCC
			R: 5'-GATACGTGGACCCTTCTGGA
10	MH2 (3)	130	F: 5'-GTTCCTTCAAGCTGCCCTATT
			R: 5'-TGAGAGAGCAAGGTATTGATTGT
11	MH2 (4)	122	F: 5'-GGTTGCACATAGGCAAAGGT
			R: 5'-TAGACAGAGAAGCTGGGCGT
11	MH2 (5)	130	F: 5'-GTGCACCTGGAGATGCTGT
			R: 5'-TGAAATCTGGGTGGAAGGAA
12	MH2 (6)	130	F: 5'-CTTTGATTTGCGTCAGTGT
			R: 5'-CAGTAGGTGGAATAGCTCCA
13	MH2 (7)	116	F: 5'-TGTCCCTCTGATGTCTTCCA
			R: 5'-GAGTTTTGTGAAAGGCTGGG

6

Combined array-comparative genomic hybridization and single-nucleotide polymorphism-loss of heterozygosity analysis reveals complex genetic alterations in cervical cancer

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BMC Genomics. 2007; 8(53)

Cervical carcinoma develops as a result of multiple genetic alterations. Different studies investigated genomic alterations in cervical cancer mainly by means of metaphase comparative genomic hybridization (mCGH) and microsatellite marker analysis for the detection of loss of heterozygosity (LOH). Currently, high throughput methods such as array comparative genomic hybridization (array CGH), single nucleotide polymorphism array (SNP array) and gene expression arrays are available to study genome-wide alterations. Integration of these 3 platforms allows detection of genomic alterations at high resolution and investigation of an association between copy number changes and expression. Genome-wide copy number and genotype analysis of 10 cervical cancer cell lines by array CGH and SNP array showed highly complex large-scale alterations. A comparison between array CGH and SNP array revealed that the overall concordance in detection of the same areas with copy number alterations (CNA) was above 90%. The use of SNP arrays demonstrated that about 75% of LOH events would not have been found by methods which screen for copy number changes, such as array CGH, since these were LOH events without CNA. Regions frequently targeted by CNA, as determined by array CGH, such as amplification of 5p and 20q, and loss of 8p were confirmed by fluorescent *in situ* hybridization (FISH). Genome-wide, we did not find a correlation between copy-number and gene expression. At chromosome arm 5p however, 22% of the genes were significantly upregulated in cell lines with amplifications as compared to cell lines without amplifications, as measured by gene expression arrays. For 3 genes, *SKP2*, *ANKH* and *TRIO*, expression differences were confirmed by quantitative real-time PCR (qRT-PCR). This study showed that copy number data retrieved from either array CGH or SNP array are comparable and that the integration of genome-wide LOH, copy number and gene expression is useful for the identification of gene specific targets that could be relevant for the development and progression in cervical cancer.

Introduction

The development of cervical cancer is a multi-step process. This process starts with infection of the epithelial layer with high-risk human papilloma virus (HPV). Cervical intraepithelial neoplasia (CIN) grade I, II and III, representing reversible stages of disease, is believed to develop towards neoplasm. From CIN I onwards genomic alterations and aneuploidy can be detected [1,2] and when disease is progressing towards CIN II and III, genetic alterations become more evident [3]. HPV E6 and E7 can induce cellular transformation due to interaction with cellular proteins involved in cell cycle control, apoptosis and genomic instability [4]. The induction of genomic instability seems to be particularly important for the establishment and development of an invasive tumor [5].

So far, different studies have investigated genomic alterations in cervical cancer by means of metaphase comparative genomic hybridization (mCGH) and microsatellite marker analysis for the detection of loss of heterozygosity (LOH). Chromosomal changes involving loss of 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q and 18q regions and gain of 1q, 3q, 5p and 8q regions at various stages of disease have been identified [1,6-8]. Some of these imbalances have been associated with decreased survival such as LOH of 18q [8] or with the transition from severe dysplasia to invasive carcinoma such as gain of 3q [9]. However, few genes, targeted by these genomic imbalances and possibly involved in carcinogenesis, have been discovered due to the lack of resolution when applying mCGH [10] whereas classical LOH studies are laborious. Array comparative genomic hybridization (array CGH) is an established high resolution method to study the whole genome for chromosomal amplifications and deletions [11]. Although array CGH has relatively few printed elements on the arrays, the strength of this technique is the high spatial resolution [12]. A limitation of array CGH is the lack of genotype information. Therefore, it does not provide information about regions of LOH without copy number alteration (CNA) such as mitotic recombination or gene conversion events. The under representation of LOH in copy number methods may be very high with up to 2/3 of LOH events being undetected because of copy-neutral and copy-gain LOH events, as was found in pancreatic cancer [13]. Recently, high-density oligonucleotide-based single polymorphism arrays (SNP arrays) have been used to identify copy number and LOH of chromosomal regions [14,15]. Appropriate software tools for the detection of CNA from SNP data are becoming available. The advantage of a combined SNP-CGH approach is the identification of allele specific gain and loss by SNP array and the robust copy number detection by array CGH.

Different factors, including changes in genome copy number, can disrupt proper gene functioning. A few studies showed correlations between chromosome CNA and gene expression changes of the affected regions [16,17]. In this study, genome-wide LOH and copy number analyses of 10 cervical cancer cell lines were investigated using SNP and CGH arrays and the sensitivity to detect CNA by the 2 platforms was compared. We aimed at identifying common regions of gains and losses and determining the influence of CNA on gene expression to find genes involved in cervical carcinogenesis.

Materials and methods

Cell lines, cultures and patient material

Human cervical cancer cell lines, HeLa, SiHa, CaSki (ATCC, Rockville, MD), CSCC1, CSCC7, CC8, CC10A, CC10B, CC11-, and CC11+ [18] were maintained in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% fetal calf serum, streptomycin and penicillin at 37°C in a humid atmosphere containing 5% CO_2. CC10A, CC10B, CC11- and CC11+ are sub clones from the same cell line, CC10 and CC11 respectively. Five normal primary cervical epithelial cell cultures (NPE) were prepared as described [19]. Two archival formalin-fixed, paraffin-embedded cervical carcinomas were obtained from the tissue bank of the Department of Pathology (LUMC, Leiden, The Netherlands).

DNA and RNA isolation

DNA from all the cultured cell lines was isolated using the salting out procedure according to Miller and Polesky [20]. Total RNA was isolated using TRIzol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) from cells that were grown to approximately 60% confluence in 250 ml culture flasks (Greiner, Frickenhausen, Germany). Total RNA was cleaned with Rneasy cleanup system columns (Qiagen GmbH, Hilden, Germany).

Array CGH

Array CGH was performed as previously described [21]. Slides containing BACs in triplicate were made at the Leiden University Medical Centre. The particular BAC set used (Welcome Trust Sanger Institute (UK)) comprises clones spaced at 1 Mb density over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis. Information regarding the full set is available in the “Cytoview” window of the Sanger Center mapping database site, Ensembl[22]. The test/reference ratios were normalized for the median of the ratios of all features. The BioConductor package Limma (Smyth and Speed 2003) version 2.6.0 was used to perform background correction with method ‘Edwards’ and within array normalization with method ‘Printtiploess’, between array normalization was not performed. After normalization the replicate spots were averaged. Probes with less than 2 valid replicate values, and probes that showed a standard deviation above 0.1 between the replicate values were excluded. Chromosomal regions demonstrating a ratio of between 1.11 and 1.41 were scored as ‘gained’ and a ratio of 1.41 or greater was scored as ‘amplified’. Chromosomal regions showing a ratio of between 0.84 and 0.73 were scored as lost whereas a second threshold for loss was set for regions showing a ratio of less than 0.73.

SNP 10K Array

Affymetrix 10K SNP array (Santa Clara, CA) containing 11,555 biallelic polymorphic sequences randomly distributed throughout the genome, excluding the Y chromosome, was used. The median distance between SNPs is ~105 kb. The average

heterozygosity for these SNPs is 0.37, with an average minor allele frequency of 0.25. DNA samples, including two control DNA samples from Affymetrix, were assayed according to the manufacturer's manual protocol (GeneChip Mapping Assay manual). Genotyping was performed using GDAS (Affymetrix, Santa Clara, CA) and copy number values were calculated using dChip version 1.3. Stretches of homozygous SNPs equal or longer than 24 calls were defined as LOH.

Gene expression microarray

Affymetrix focus arrays (Santa Clara, CA), representing 8,793 human sequences from the NCBI Refseq database, were used for mRNA expression profiling according to the manufacturer's instructions. Data files of all microarrays were normalized using the Robust Multi-Chip Average (GCRMA) method[23]. Quality control was performed on the arrays using Affy-Probe Level Model (AffyPLM) [24]. Microarrays for each cell line were performed in duplicate. The signal intensities of the replicates were averaged after normalization. To identify differentially expressed genes between cell lines with and without gain, amplification or deletion of a chromosome arm or region, unpaired two-sample t-tests were performed.

Integrative analysis

To compare the data from the 3 high dimensional techniques; array CGH, SNP array, and expression array, the copy number and expression values were transformed to contain log 2, median centered values. Access to complete microarray data sets will be available at NCBI Gene Expression Omnibus. Normalization of array CGH data and expression arrays resulted in values that are equivalent to log2 intensity values. The data retrieved from dChip version 1.3 from the SNP arrays showed a copy number of 2 for unaffected probes on a linear scale. These data were divided by 2 and log2 transformed. A per gene median centering to zero was performed. Array CGH smoothing was performed using a smoother that retains sharp transitions between regions with genomic alterations. The correlation between gene expression and physical genomic alterations was determined by computing the Pearson correlation between the expression of each gene to the smoothed array CGH value of the nearest BAC on the chromosome. The resulting p-values were subjected to the Benjamini and Yekutieli false discovery rate procedure [25]. The data retrieved from dChip version 1.3 from the SNP arrays showed a copy number of 2 for unaffected probes on a linear scale.

Fluorescent in situ hybridization (FISH)

Metaphase preparations of all cell lines were obtained using colcemid according to standard procedures [26]. BAC probes for the genes selected on chromosome 5, chromosome 8 and chromosome 20 (Table 1) were ordered from the BACPAC Resource Centre at Children's Hospital Oakland Research Institute (Oakland, CA). All BAC probes were fluorescein-12-dUTP- or digoxigenin-12-dUTP-labelled (Roche, Basel, Switzerland). The following centromere probes were used: the pG-A16 probe for centromere 5; the D8Z2 probe for centromere 8; and the p3.4 probe for chromosome 20. All centromere probes were kindly provided by J. Wiegant

Table 1. Aberrations determined by FISH analysis at chromosomes 5, 8 and 20.

Gene	Location	BAC clone	1	7	8	10a	10b	11-	11+
SKP2	5p13.2	RP11-624K2	-	-	-	-	-	-	-
TRIO	5p15.2	RP11-1150G22	-	-	-	-	-	-	-
hTERT	5p15.33	RP11-117B23	-	-	-	-	-	-	-
GDNF	5p13.2	RP11-914H3	-	-	-	-	-	-	-
ANKH	5p15.2	RP11-99I1	-	G	-	G	G	G	-
TUSC3	8p22	RP11-44L18/184C1	-	-	-	L	-	L	L
ZNF-217	20q13.2	RP4-724E16	G	-	G	G	G	-	-
CYP24A1	20q13.2	RP5-1075G21	G	-	G	G	G	-	-
MYBL2	20q13.12	RP11-153L9	G	-	G	G	G	-	-
AIB1	20q12	RP11-1151C1	G	-	G	G	G	-	-

(-) no aberration; G - gain; L - loss. Cell lines: 1- CSCC1; 7- CSCC7; 8 - CC8; 10a - CC10A; 10b - CC10b; 11 - CC11-; 11+ - CC11+; S - SiHa, C - CaSki , H- HeLa.

(Department of Molecular and Cellular Biology, LUMC) and labelled with biotin-16-dUTP (Roche, Basel, Switzerland). Triple-colour FISH experiments were performed as previously described [27]. Centromere and BAC signals of 20 metaphases were analyzed per probe combination per cell line.

Microsatellite analysis

Genomic DNA was isolated from flow-sorted tumor cell subpopulations derived from formalin-fixed, paraffin-embedded cervical carcinomas as previously described [28]. Eight fluorescein-labelled primer pairs of microsatellite markers comprising MOGC, D6S265, C125, TY2A, TNF, D6S273, D6S294 and D6S1666 [29], spanning altogether 26 MB from 6p22.1 to 6p12.1, were used. PCR, electrophoresis and analysis was performed as described previously [30]. Comparing normal and tumor DNA, a reduction of more than 50% of one allele was assigned as complete LOH.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described previously [31]. Expression of the genes of interest was normalized by geometric averaging of multiple internal control genes (CPSF6, HNRPM, EEF1A1, RPL11 and RPL13) using the Genorm program [32]. Out of 5 normalization genes the best 3 were selected using this program; EEF1A1, RPL13 and RPL11. Primer sequences for RPL11 are 5'-ACTTCGCATCCGCAAACTCT (FWD) and 5'-AAGGTGTTGGAGCAGXTCACA (REV). Primer sequences for EEF1A1, RPL13 were reported previously [31]. Commercially available primers were used for TUSC3, ANKH and TRIO (Superarray, Frederick, USA). Primer sequences for SKP2 are: 5'-CCTATCACTCAGTCGGTGCTATGA (FWD) and 5'-AATCGTGCCAGATGGTAC-CCT (REV). To determine differential gene expression between normal cervical epithelial cell cultures (NPE), cell lines with and without deletion, gain or amplification, one-way ANOVA was performed.

Results

Genome-wide LOH and copy number analyses of 10 cervical cancer cell lines were investigated using SNP and CGH arrays to identify common regions of amplification. Also the two platforms were compared in their sensitivity to detect CNA. Furthermore, data from gene expression arrays were combined with array CGH data to investigate an association between gene expression and copy number.

CGH and SNP-LOH analysis

Various genomic alterations affecting the majority of chromosomes were found in cervical cancer cell lines. The CGH findings for all amplifications, gains and losses detected in these cell lines are displayed in Figure 1A. Gains or amplifications of 5p, 5q, 8q, 9q, 17q and 20q occurred in most of the cell lines. Physical loss of chromosomal regions at 4p, 8p, 11q, 13q and 18q, occurred in at least half of the cell lines. Regions frequently targeted by gain or loss with small overlapping regions were present at 8p, 8q, 9p, 11q and 17q. The minimal region of overlap at 8p23 involved *TUSC3*, a tumor suppressor gene that showed loss in 5 cell lines. At 8q24, the minimal region of overlap included *c-Myc* which showed gain in 8 out of 10 cell lines. Amplification at 9q33-q34 included *endoglin*, a gene involved in tumor angiogenesis and predictive for metastasis in cervical cancer [33]. Loss at 11q25 involved 2 members of the IgLON family of cell adhesion molecules, *OPCML* and *HNT*. The minimal region of overlap at 17q11-q21 included *ERBB2* and *TOP2A*, genes that are amplified in breast cancer [34]. As for SNP-LOH, most chromosome arms were affected by LOH in the cell lines (Figure 1B). More than 5 overlapping regions of LOH were evident for chromosome region 5q, 6, 8p, 10q, 11q, 14q, 18 and 20p.

Comparison between SNP and BAC microarray

Concordance between array CGH and SNP array in the detection of CNA was investigated. Overall, the BAC and SNP arrays identified exactly the same regions of gain in 91% of cases, when taking an average of all cell lines (Table 2). Similarly, the detection of the same regions with loss between the 2 techniques was on average 94% (Table 2). Subsequently, we investigated the overlap between copy number loss and LOH and copy number gain and LOH for both platforms. For LOH to occur, measured as a reduction in copy number, physical loss is required. Thus all cases with copy number reductions are expected to have undergone LOH. In case of physical loss, around 60% was observed to encompass regions of LOH (Table 3). Overall, copy number reductions as measured by array CGH showed a higher overlap with LOH than SNP array (Table 3), namely 66% for array CGH and 57% for SNP array, when taking an average of all cell lines. The occurrence of physical gain involves a change in copy number and thus a change in the proportion of genotypes which would imply a call for LOH. Around 28% of copy number gain was found to cover regions of LOH (Table 3) and frequencies were comparable between SNP array and array CGH. Also, the overall percentage of LOH in copy neutral, copy gain and copy loss events was investigated for each cell line. The average frequency

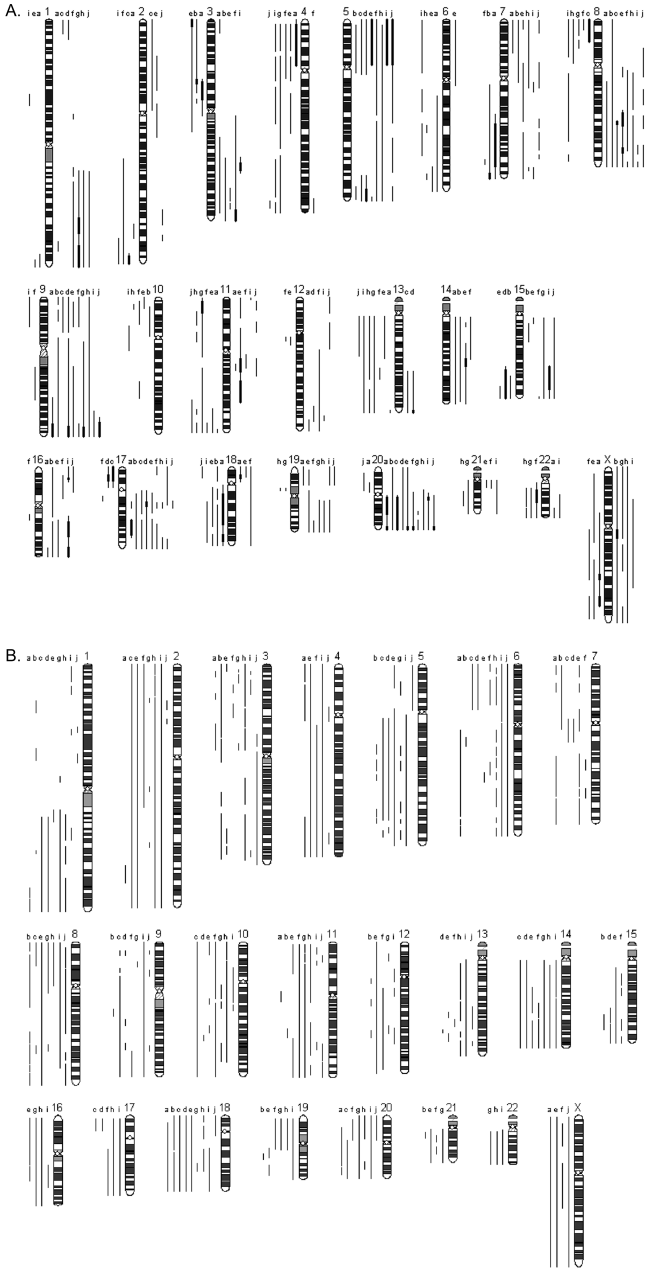


Figure 1. Chromosomal gains and losses in cervical cancer cell lines. A) Array CGH results are depicted by lines to the right of the chromosomes as gains and on the left as losses. Thick solid lines represent regions with amplifications of more than 1 copy loss. B) SNP-LOH results show LOH as lines on the left side of the chromosomes. The letters above the lines indicate the cell lines: a) CSCC7, b) CSCC1, c) CC11-, d) CC11+, e) CaSki, f) CC8, g) CC10b, h) CC10a, i) SiHa and j) HeLa.

Table 2. Percentage of gains and losses, determined for array CGH and SNP array data.

	Gains			Losses		
Cell line	**SNP (%)**	**CGH (%)**	**Con. (%)**	**SNP (%)**	**CGH (%)**	**Con. (%)**
CSCC1	20,6	26,9	88,3	14,9	10,4	94,6
CSCC7	16,9	16,4	93,9	16,7	12,3	95,4
CC8	35,7	30,2	88,6	12,5	11,3	89,6
CC10A	10,3	23,5	86,9	19,7	8,2	83,4
CC10B	15,9	11,2	94,4	21,7	17,4	95,4
CC11-	16,4	13,9	92,9	2,4	1,8	99,3
CC11+	11,0	14,5	93,9	3,5	1,8	98,3
CasKi	20,7	30,5	87,6	22,6	13,8	91,1
SiHa	12,6	13,7	92,7	23,1	20,9	94,1
HeLa	30,3	24,0	86,2	5,4	3,9	98,2
Average	19,0	20,5	90,5	14,3	10,2	93,9

The percentage of concordance between both techniques is shown in the latter column of gains and losses. Abbreviations: Con. is concordance.

Table 3. Percentage of overlap between LOH and copy number gains and losses.

	% overlap between LOH and CNA			
	Gains		Loss	
cell line	**SNP (%)**	**CGH (%)**	**SNP (%)**	**CGH (%)**
CSCC1	41,3	30,2	39,4	52,1
CSCC7	13,1	14,9	72,0	92,0
CC8	17,8	16,9	4,8	18,2
CC10A	26,6	15,7	73,9	82,2
CC10B	22,1	35,4	45,6	42,4
CC11-	32,3	38,9	77,3	90,1
CC11+	9,1	6,9	57,2	87,5
CasKi	60,8	52,4	52,4	45,7
SiHa	38,1	49,6	95,7	97,0
HeLa	19,5	15,8	46,6	54,8
Average	28,1	27,7	56,5	66,2

of LOH from all cell lines was 21-26% for loss, 17% for gain and 60% for copy neutral events (Table 4). Although array CGH and SNP array are comparable in the level of detection of copy number gain and loss, alterations in regions of LOH were often not found by copy number analysis because these LOH changes did not result in CNA (Figure 1B and Table 4). For example, 6 of 10 cell lines showed LOH of 6p while array CGH detected physical loss of 6p in only one cell line. This suggests that the other 5 cell lines underwent mitotic recombination upon loss of one arm, thereby not affecting copy number. Examples of complex genomic alterations are shown for chromosome 8 and 20 of SiHa and chromosome 3 of CaSki (Figure 2). In these examples (Figure 2A and B), LOH is caused by loss of one chromosome,

Table 4. Percentage of LOH determined for copy neutral, copy gain and copy loss events.

	% LOH				
	Copy neutral		Gain		Loss
Cell line	SNP (%)	CGH (%)	SNP (%)	CGH (%)	SNP (%)
CSCC1	25,7	30,4	43,9	41,8	30,3
CSCC7	17,5	19,8	12,8	14,1	69,6
CC8	79,1	78,5	19,0	15,3	1,7
CC10A	62,7	77,6	5,9	7,9	31,3
CC10B	52,3	59,6	12,5	14,2	35,2
CC11-	80,1	80,7	14,7	14,9	5,2
CC11+	83,6	86,1	5,5	5,5	10,9
CasKi	53,4	60,0	20,5	26,6	26,0
SiHa	54,6	54,2	8,1	11,5	34,3
HeLa	63,3	74,1	25,8	16,6	10,9
Average	57,2	62,1	16,9	16,8	25,5

accompanied by duplication of the remaining chromosome and subsequent deletions and translocations. The copy number data from SNP array as deduced from the dChip data (in blue lines) and array CGH (red lines) clearly follow the same pattern in these examples, supporting their compliance.

Correlation between DNA copy number and gene expression

To correlate the chromosomal changes to alterations in gene expression profiles, we have searched for changes in the expression of genes located in the regions targeted by chromosomal gains and deletions. This was done by combining gene expression data from Affymetrix focus arrays with array CGH data. The average gene expression values of all cell lines were used as a reference for each cell line to determine increases or decreases in gene expression. Genome-wide, no correlation was observed between copy-number and expression. Since 5p, 8p23, 8q24, 9q33-q34, 11q25 and 20q are regions with many alterations, we focused on these areas. For 20q we compared cell lines harboring amplifications with cell lines containing gains since no cell line was devoid of 20q gain or amplification but we did not find significant differences in gene expression. Neither did we find significant changes in gene expression at 8p23, 8q24 and 11q25. At 9q33-q34, cell lines with amplification (CSCC7, CC11+, CC10A, CC10B, SiHa and HeLa) showed a significantly higher expression compared to cell lines with gains or no CNA correlation for 9 out of 86 genes (10%); *POMT1*, *FLJ35348*, *LHX3*, *NDOR*, *SH3GLB2*, *ST6GALNAC4*, *CDK9*, *PTAN1* and *TRAF2* ($p < 0.05$, T-test), of which the latter 3 have been implicated in tumorigenesis. Fourteen of 64 genes (22%) at 5p showed a significantly higher expression in cell lines with amplification (CaSki, SiHa and HeLa) compared to cell lines with gains or no CNA (Table 5).

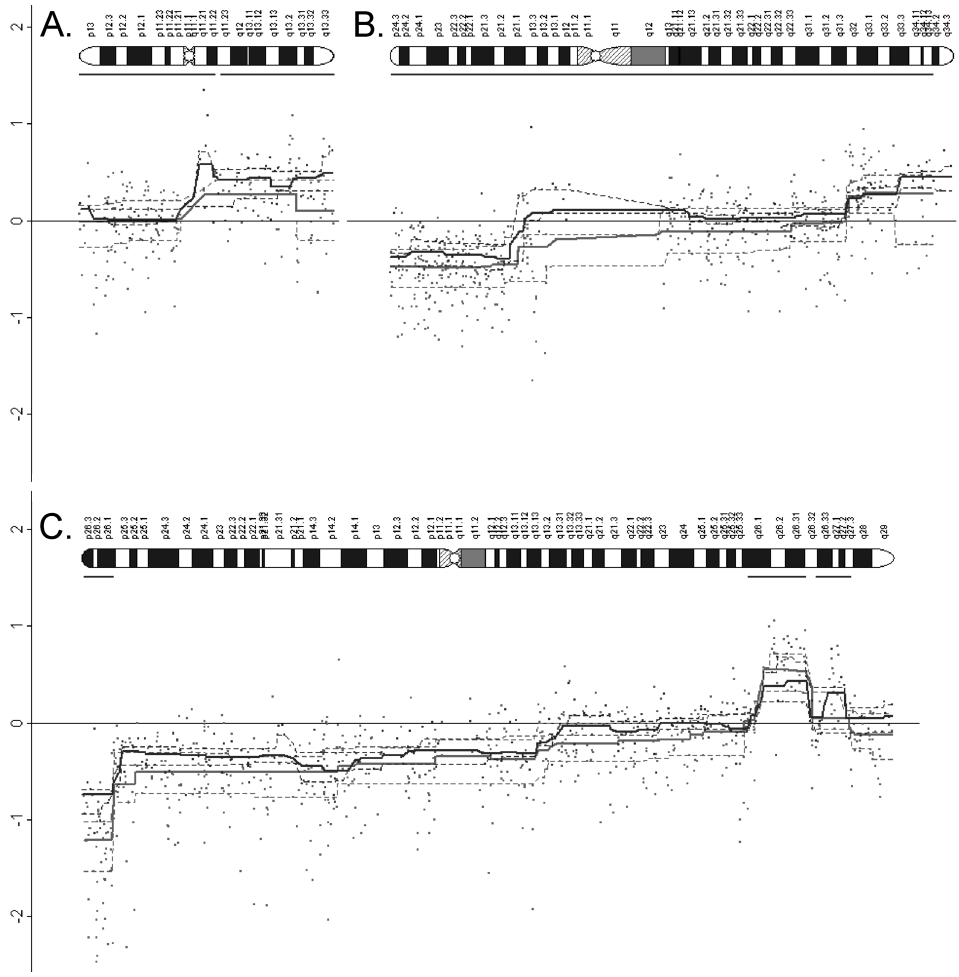


Figure 2. Complex genomic alterations of chromosome 20 (A) and chromosome 8 (B) of SiHa and chromosome 3 of CaSki (C). Below each chromosome ideogram, SNP array data are shown in blue and array CGH data in red. The blue line directly beneath the ideogram depicts LOH, whereas the blue line shifting around the baseline (0) shows copy number retrieved from SNP array data. The dotted blue and red lines represent the confidence intervals for the copy number of the SNP and the CGH array, respectively.

Validation of genetic alterations by FISH and LOH

The DNA copy number retrieved from the array CGHs was validated by FISH. Based on the gained, amplified or lost regions of 5p, 20q and 8p, BACs were selected that covered potential oncogenes or tumor suppressor genes. FISH analysis on all cell lines was performed using the BAC clones encompassing *SKP2*, *TRIO*, *hTERT*, *GDNF* and *ANKH* genes on 5p, *TUSC3* on 8p and *ZNF-217*, *CYP24A1*, *MYBL2* and *AIB1* on 20q (Table 1). Besides additional copies of the area covered by BACs at 5p, other complex genetic alterations such as translocations and inversions were

Table 5. Genes significantly overexpressed in cell lines with amplification of 5p (T-test, $p < 0.05$).

Gene symbol	Chromosomal Location
ARSB	chr5p11
RPL37	chr5p13
SKP2	chr5p13
OSMR	chr5p13.1
TARS	chr5p13.2
RAD1	chr5p13.2
SUB1	chr5p13.3
RNASEN	chr5p13.3
POLS	chr5p15
SDHA	chr5p15
TRIO	chr5p15.1-p14
NDUFS6	chr5p15.33
TRIP13	chr5p15.33
PDCD6	chr5pter-p15.2

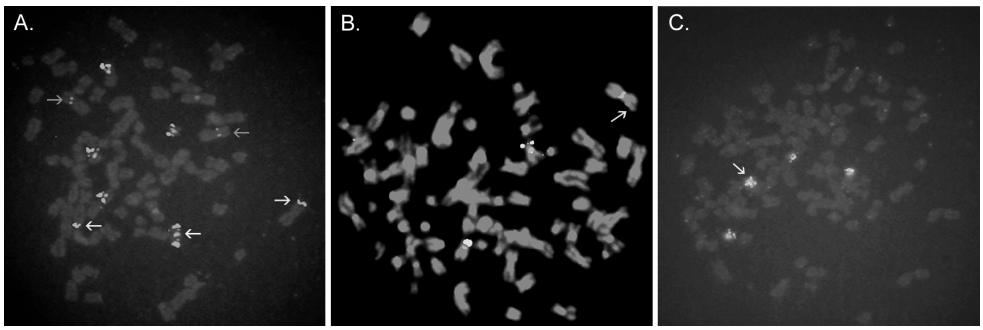


Figure 3. FISH of metaphase preparations depicting copy number alterations. A) CaSki - 5 chromosomes 5 (centromere 5-blue; TRIO-green; SKP2-red); one isochromosome 5p (yellow arrow), 2 translocations of RP11-1150G22, encompassing TRIO (green arrow) and 2 translocations of RP11-624K2, encompassing SKP2 (red arrow). B) CaSki - 3 chromosomes 8 (centromere 8-red, TUSC3-green) with loss of one copy the BACs encompassing TUSC3, RP11-44L18/184C1 (green arrow). C) HeLa - 4 chromosomes 20 (centromere 20-blue, ZNF-217-red, CYP24A1-green); one isochromosome 20q (yellow arrow).

detected (Figure 3A). *TUSC3* is a putative tumor suppressor gene at 8p that is covered by 2 overlapping BAC clones, RP11-44L18 and RP11-184C1 BAC. Loss of the area at 8p was concordant between array CGH and FISH for CC10A, CC11- and SiHa (Figure 3B). Signals for BACs at chromosome 20q were equal to the number of centromere signals or showed additional copies (Table 1 and Figure 3C), indicating that the whole arm or the chromosome was gained, which is in agreement with the array CGH data (Figure 1A). Overall, FISH assays confirmed the accuracy in which DNA CNA were detected by array CGH for these loci and highlighted the gains and

amplifications of the *ZNF-217*, *CYP24A1*, *MYBL2* and *AIB1* genes at 20q, *SKP2*, *TRIO*, *hTERT*, *GDNF* and *ANKH* genes at 5p and the loss of the *TUSC3* gene at 8p. LOH of 6p detected by SNP array in cell line CC11-, CC11+ and CC10A was confirmed by LOH analysis of the tumors from which the cell lines originate, using microsatellite markers (Figure 4).

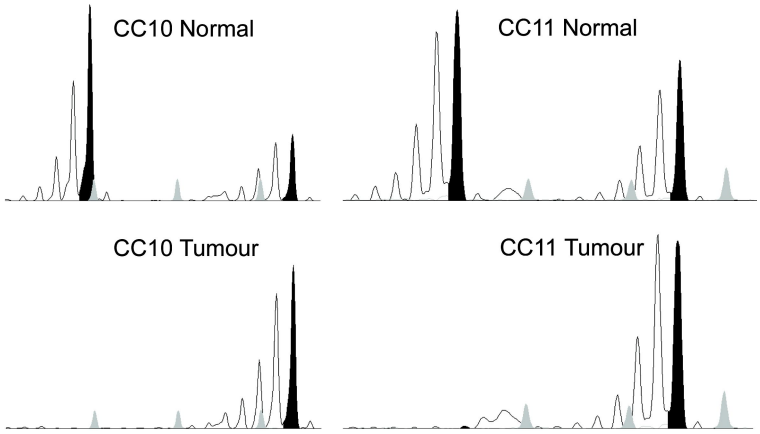


Figure 4. LOH at 6p21.3 is demonstrated for microsatellite marker C125. Tumor samples from which cell lines CC11 and CC10 originated were analyzed for LOH. In black the particular alleles are depicted and in grey the marker is shown.

Validation of gene expression by qRT-PCR

To validate the microarray data, upregulated expression of 3 potential oncogenes (*SKP2*, *ANKH* and *TRIO*) were analyzed by qRT-PCR. These genes were located on 5p that was shown to be gained or amplified by CGH in half of the cell lines. Microarray data showed upregulation of *ANKH* in 2 of the 3 cell lines with amplification of 5p which did not reach statistical significance. Five normal cervical epithelial cell cultures (NPE) were included in the analysis to investigate the difference in expression level between normal and tumor cells, with and without amplification, from the cervix. Expression was significantly different between cell lines with 5p amplification, cell lines with 5p gains or no 5p alterations, and NPE (Figure 5). At chromosome arm 8p, deletion of the tumor suppressor gene *TUSC3*, as found by FISH analysis (Table 1 and Figure 3B), was evident in CaSki, SiHa, CC10A, CC11- and CC11+. No probe set on the expression array was available for *TUSC3*. Therefore, qRT-PCR was carried out on all cell lines. Only SiHa and CaSki showed a substantial drop in expression, compared to the expression levels of the other cell lines and NPE (data not shown).

Discussion

The present study describes genome wide LOH and copy number analyses of 10 cervical cancer cell lines using SNP and CGH arrays to identify common regions of

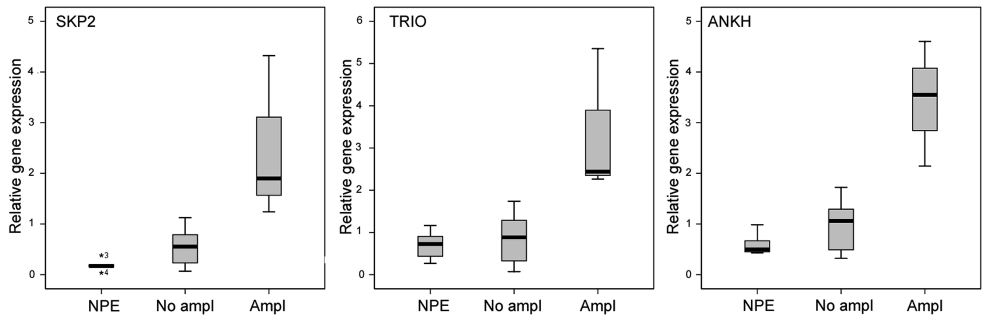


Figure 5. Box plots show differences in gene expression measured by qRT-PCR of SKP2 ($p = 0.003$, ANOVA); TRIO ($p < 0.001$, ANOVA) and ANKH ($p < 0.001$, ANOVA) in cell lines with and without amplification of 5p and normal cervical epithelial cell cultures (NPE). Ampl: amplification.

amplification and deletion. Also, the sensitivity to detect CNA by the 2 platforms was compared. Furthermore, integrated copy number and gene expression analyses were performed to investigate an association between CNA and gene expression.

CNA retrieved from array CGH and genotype information analyzed from SNP arrays revealed very complex large-scale changes. Chromosome arms that were found to be gained and/or amplified in most of the cell lines by array CGH in this study were 5p, 8p, 8q, 9p, 9q, 17q and 20q. Especially gains of 5p, 8q and 20q have been frequently reported in cervical cancer [6,7,9,35-39]. Also the 1q and 3q arms, often found to be gained [7,9,35,38-40], were overrepresented in 5 of the 10 cell lines. Gains of 17q and chromosome 9 are less consistently reported [6,36]. Gain of 8q24, present in 8 cell lines, included *c-Myc* in the minimal region of overlap. Amplification of the *c-Myc* oncogene is a frequent event in cervical tumors, found in 25% to more than half of the tumors [41,42]. Chromosome arms affected by loss in half, or more, of the cell lines were 4p, 8p, 11q and 13q of which especially 4p and 13q have been reported regularly in cervical cancer [7,9,36,39,40,43]. Six cell lines showed loss of 11q25 that includes *OPCML* in the minimal region of overlap. *OPCML* is a tumor suppressor gene in epithelial ovarian cancer [44]. Although the genotype data have to be interpreted with some caution because of the use of cell lines, many of the frequent changes that we observed were in concordance with previous studies.

The SNP-LOH array data showed that most chromosome arms were affected by LOH in the cell lines. More than 5 overlapping regions of LOH were evident for chromosome region 5q, 6p, 6q, 8p, 10q, 11q, 14q, 18p, 18q and 20p. LOH of the majority of these chromosomes, specifically 5q, 6p, 8p, 10q, 11q and 18, has been reported previously as frequent events in cervical cancer [45-47]. Whereas LOH of 8p resulted in copy number loss, LOH of 6p did not result in CNA for most cases, indicative of mitotic recombination events.

A comparison between CGH and SNP array revealed that the overall concordance in detection of the same areas with CNA was on average 91% for gain and 94% for loss (Table 2). Thus both platforms are comparable in the detection of particular regions with gain or loss. Additionally, the use of high-density SNP arrays showed

that cervical cancer cell lines harbor multiple regions of LOH which are not detected by methods which screen for CNA such as array CGH. Accordingly, comparison of array CGH and SNP array in genome-wide analysis shows that detection of CNA by SNP array is reliable and has the added advantage of providing genotype information. However, LOH was not consistently detected by SNP array because many copy number changes did not result in true reduction to homozygosity since most of the cervical cancer cell lines are multiploid (Figure 2C and Table 3). As expected, the areas with gain and amplification showed less LOH than regions with deletion. The overlap between copy-reduction regions and LOH was 10% higher for array CGH than for SNP array (Table 3), which suggests that array CGH is slightly better in assigning the correct regions for CNA. Although some regions of LOH are missed, thereby particularly underestimating the amount of LOH in areas with gain and amplification, an estimation was made for the distribution of LOH in regions with loss, gain and copy-neutral regions. We found that copy-neutral and copy-gain regions accounted for around 75% of all LOH identified (17% represented copy-gain LOH) while LOH associated with copy-reduction occurred at a rate of about 25% (Table 4). These percentages are similar to a recently published paper from Calhoun et al. that described only 32% of LOH events to be associated with copy-reduction [13]. Further improvement in the data from the SNP arrays may be possible using higher density SNP arrays, thereby increasing resolution and the ability to assess subtle copy number changes.

Genome-wide, we did not find a correlation between copy-number changes and expression. Lack of an association may be due to the different histological types from which the cell lines originate (Squamous carcinoma, Adenocarcinomas and Adenosquamous carcinomas) with complex genetic aberrations. Besides increased DNA dosage which may result in overexpression, transcription is influenced by other factors such as the presence and quantity of transcription and repressive factors; CpG methylation; histone methylation and acetylation status, and possibly microRNA's [48]. Thus, amplification or deletion on its own does not have to be a prerequisite for a change in gene expression. Amplification of 5p, however, correlated with a significant higher gene expression for 22% of the genes, including *SKP2*, *TRIO*, *OSMR*, *RPL37* and *PDCD6* (Table 5). The latter genes were previously reported in cancer, either associated with increased expression or with growth stimulatory properties [49-53].

Array CGH findings of CNA at 5p, 8p and 20q were confirmed by FISH analysis. Gains and amplifications at 5p, as found by array CGH, were validated by BACs covering candidate oncogenes, *SKP2*, *TRIO*, *ANKH*, *GDNF* and *hTERT*. *SKP2* is important for cell cycle progression and its inhibition decreases proliferation of tumor cells [54]. Besides gain of the region covering the *SKP2* gene, we found a significant increase in expression between normal cervical epithelial cells, tumor cells without amplification and tumor cells with amplification. Correlation between amplification of chromosome 5p and increased expression of SKP2 was previously shown in HPV-immortalized keratinocytes [55]. Upregulated expression of *SKP2*, *ANKH* and *TRIO*, as determined by qRT-PCR in cell lines with 5p amplification, confirmed gene expression data from Affymetrix focus arrays. *TRIO*, which has a putative role in cell

cycle regulation, was found to be amplified and highly expressed in bladder cancer [50] that associated with proliferation and invasive phenotype. *ANKH*, which is involved in tissue calcification [56], was found to be amplified in bladder cancer [50] and small cell lung cancer cell lines [57]. Thus, upregulation of expression through amplification may be the case for *TRIO*, *ANKH* and *SKP2* in some neoplasms, including cervical cancer, as indicated by our data. This may hold for *hTERT* and *PRKAA1* as well since it was reported that amplification at 5p in cervical cancer correlated with increased gene and/or protein expression [58,59]. The telomeric region at 8p includes a candidate tumor suppressor gene, *TUSC3* [60,61]. However, physical loss of this region did not correlate with a decrease in gene expression of *TUSC3*, as determined by qRT-PCR. All cell lines showed gains or amplifications of 20q, a chromosome arm which is often amplified in cancer, including cervical cancer [9,36,38,62]. At 20q increased copy numbers, detected by array CGH, were confirmed by FISH. Candidate oncogenes, *ZNF-217*, *CYP24A1*, *MYBL2* and *AIB2*, encompassing the region analyzed by FISH, were previously reported to be amplified in other types of cancer as well [63-65]. However, no significant difference in gene expression was found between tumors with gain and tumors with amplification of 20q. Also in oesophageal adenocarcinoma, no correlation was detected between amplification of *ZNF-217*/ *CYP24A1* at 20q and increased mRNA expression [66].

From this study we conclude that copy number data retrieved from either array CGH or SNP are comparable. The SNP array provided a reliable copy number as well as genotype information, which was suitable for the analysis of complex genetic alterations present in cervical cancer cell lines. Frequently occurring gains, amplifications and deletions on chromosome arms 5p, 20q and 8q were verified by FISH. Amplification at 5p associated with overexpression of candidate oncogenes. Accordingly, the possibility to integrate genome-wide LOH, copy number and gene expression is useful for the identification of gene specific targets that could be relevant for the development and progression in cervical cancer.

Acknowledgements

The study was supported by the Dutch Cancer Society, grant no. RUL2001-2465.

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7

Summary and General Discussion

Immune surveillance is of utmost importance in preventing cervical carcinogenesis. Cytokines play a central role in directing and fine tuning the immune response. In cancer, cytokines can either be involved in stimulating the anti-tumor immune response or in tumor growth and progression. Certain cytokines play a dual role. For example, TGF-β acts as a tumor suppressor early in carcinogenesis and as a tumor promoter late in carcinogenesis. The studies presented in this thesis concern molecular analyses of immune escape mechanisms besides HLA class I loss, such as loss of TNFα expression and TGF-β insensitivity in cervical cancer. Furthermore, we studied genome-wide molecular alterations to identify possible genes involved in cervical carcinogenesis, including genes involved in the immune response. In this chapter, the results obtained are summarized and put into perspective.

The primary cause for the development of cervical cancer is infection with human papillomavirus (HPV). However, the majority of HPV infections and cervical intraepithelial neoplasia (CIN) are cleared by cellular mediated immune responses [1,2]. A deterioration of the immune status of a person likely predisposes to persistence of infection, the formation of CIN lesions and a higher risk for the development of cancer [3]. A well-known strategy of neoplastic cells to evade cellular mediated immunity is loss of antigen presentation via human leukocyte antigens (HLA) class I [4]. Nevertheless, some cervical tumors escape immune surveillance without loss of HLA class I [5].

To investigate the immune escape mechanisms utilized by HLA positive tumors, in **Chapter 2** the gene expression profile of cervical tumors with no/minimal loss, partial loss and total loss of HLA class I, measured by microarray, is described. A significant difference in expression of 150 genes was shown between tumors with total loss and no/partial loss of HLA class I. The Gene Ontology (GO) biological process that encompasses most of these genes is defense response. HLA positive tumors showed a significant increase of genes involved in proinflammatory and acute phase response. When a difference in the extent of infiltrate between the 2 groups (HLA negative and HLA positive tumors) was included as a possible confounder in the analysis, defense response, including proinflammatory and acute phase response, remained significantly higher in HLA positive tumors. Besides genes involved in an inflammatory response also a number of interferon γ (IFNγ) induced genes were upregulated in HLA positive tumors. A possible reason for this phenomenon would be that immune cells attracted by the tumor become activated by the presentation of viral and/or tumor-derived peptides and start producing IFNγ. IFNγ may also activate tumor associated macrophages (TAMs) which in turn start producing proinflammatory cytokines and chemokines [6,7]. In this scenario the question remains how cells from the immune system in HLA positive tumors become activated. We speculate that in contrast to HLA negative tumors, activated cytotoxic T lymphocytes present in the tumor destroy HLA positive tumors, resulting in debris. This debris is taken up by antigen presenting cells (APC), resulting in activation of the APC. Then, these APC present (viral) antigens to T helper lymphocytes which in turn become activated and start producing cytokines and interferons. Although this sequence of reactions would sustain an anti-tumor response [8], apparently this immune response is not sufficient

to eradicate the tumor. A recent paper by de Boer et al. showed a strong positive correlation between HLA class I expression and HPV-specific immune response [9]. This observation emphasizes the importance of HLA class I expression in maintaining anti-tumor immunity.

Although proinflammatory cytokines and chemokines play an important role in attracting and stimulating immune cells, these molecules may also contribute to tumor development by functioning as tumor growth factors or by stimulating angiogenesis [10,11]. In cervical cancer, expression of the CCL2 (also known as MCP-1), a chemoattractant of monocytes, associated with the presence of TAMs and a decreased overall survival [12], suggesting a role for this chemokine in tumor progression.

Since cytokines are pleiotropic and work in concert, it is difficult to predict whether the proinflammatory environment will benefit the tumor or will sustain an anti-tumor immune response [13,14]. This will also be dependent on tumor type and stage. According to our results, genes associated with defense response that differed significantly in expression between HLA positive and HLA negative tumors, encoded a number of chemokines and 2 acute phase proteins. Especially the serine protease inhibitors SerpinA1 and SerpinA3 showed a major difference in gene expression. High SerpinA1 and SerpinA3 expression in HLA positive tumors was confirmed by real-time PCR and immunohistochemistry. Other serine protease inhibitors such as PAI-1, maspin and headpin can exert tumor inhibiting or tumor promoting activity [15-17]. In cervical cancer, PAI-1 expression in the tumor was shown to be a prognostic unfavorable factor [18]. This may be due to its stabilizing effect on matrix composition which may facilitate the tumor by providing growth factors and supporting angiogenesis [17,19]. In inflammatory processes, SerpinA1 and SerpinA3 protein levels are often increased in plasma [20]. Studies have shown that SerpinA1 acts as a potent anti-inflammatory agent by inhibiting LPS-induced macrophage activation *in vitro* and suppressing nuclear transcription factor-Kappa B translocation [21-23].

We observed that protein expression of either SerpinA1 or SerpinA3 in HLA positive tumors correlated with poor prognosis. Previously, high protein expression of SerpinA1 in colorectal and lung adenocarcinomas was shown to be a poor prognostic factor [24,25]. SerpinA3 protein expression has been detected in gastric and hepatocellular carcinoma [26,27]. Possible mechanisms by which SerpinA1 and SerpinA3 could be involved in tumorigenesis are still poorly understood. It has been suggested that SerpinA1 plays a role in immune suppression since it caused strong inhibition of NK cytotoxicity *in vitro* [28]. In accordance with this observation, SerpinA1 was shown to inhibit granzyme B, a trypsin-like protease [29]. Thereby, SerpinA1 could restrain anti-tumor immunity through inhibition of the cytotoxic potential of immune cells. In the case of SerpinA3, inhibition of apoptosis is a suggested mechanism by which tumorigenesis could be promoted. In hepatoma cells, SerpinA3 inhibited apoptosis by inactivating chymotrypsin [30]. Since HLA positive tumors might be targets for cytotoxic T lymphocytes, expression of the SerpinA1 and/or SerpinA3 by the tumor cells may provide the tumor with an advantage by promoting survival and/or immune suppression. However, when not taking HLA status into account, SerpinA1 or SerpinA3 did not significantly affect survival or clinical parameters. Since SerpinA1 and SerpinA3

participate in a large network of cytokines, chemokines and other immune regulatory mediators, it is conceivable to assume that these Serpins have a limited influence on the final outcome of a tumor promoting or tumor inhibiting environment.

Tumor necrosis factor α

Tumor necrosis factor α (TNFα), a cytokine with strong proinflammatory properties was demonstrated to be expressed in normal cervical epithelium, but not in most CIN and only in about 50% of cervical tumors [31]. The TNFα gene is located at chromosome region 6p which is affected by LOH in about 50% of cervical tumors [5]. Based on these findings, we hypothesized that *TNFα* could be a classical tumor suppressor gene. To investigate whether loss of TNFα, as a mechanism to escape immune clearance, is due to (epi)genetic inactivation of TNFα, loss of *TNFα* expression was studied in association with (epi)genetic characteristics in **Chapter 3**. *TNFα* mRNA expression, as studied by RISH, was found in 55% of tumors, which is in accordance with previous data [31]. However, no significant association was observed between loss of *TNFα* expression and genetic inactivation, as studied by microsatellite analysis of the respective locus and mutation analysis. Epigenetic inactivation by means of CpG promoter methylation did not reveal a correlation with lack of *TNFα* expression. Whereas our study shows that genetic inactivation as a cause for loss of *TNFα* expression is unlikely, we cannot exclude that promoter and exon 1 CpG demethylation is a prerequisite for transcription but insufficient on its own. Dependence on several epigenetic factors such as histone acetylation, histone methylation, and DNA demethylation, either acquired during cell differentiation and/or stimulation [32] and/or presence of tissue and stimulus-specific transcription factors may determine the outcome of *TNFα* mRNA expression in cervical epithelium [33, ,34]. Furthermore, we investigated whether the TNF2 allele, which has been associated with upregulated TNFα expression, was preferentially targeted by LOH. Our data did not reveal preferential loss of the *TNF2* allele, nor did we observe an association with high *TNFα* expression in cases carrying the *TNF2* allele. Eight polymorphisms have been described within the *TNFα* promoter which could affect the binding of transcription factors, influence the activity of the promoter and resulting RNA and protein levels [35]. The functional significance of these polymorphisms remains to be elucidated. As for the *TNF2* allele, characterized by the -308 G/A *TNFα* gene promoter polymorphism, conflicting results were obtained with studies associating this polymorphism with disease susceptibility [35]. In cervical cancer, 3 out of 4 studies did not observe an association of the *TNF2* allele with cervical cancer, which is in accordance with our data [36-38]. Thus *TNF2* does not seem to play a role in cervical cancer.

Besides regulation of *TNFα* expression, the question remains what role TNFα plays in cervical cancer. Nowadays, development and progression of a number of cancers is thought to be driven largely by chronic inflammation [39] of which TNFα is an important mediator [40,41]. However, in contrast to most tumors, the primary cause for development of cervical cancer is persistent infection with high risk HPV. HPVs are well able to subvert the immune response [42,43]. There is evidence that an inflammatory environment is suppressed and an immunosuppressive environment is

stimulated by relative downregulation of TNFα and upregulation of IL-10 in CIN compared to normal cervix [44]. Thus TNFα, which may interfere with HPV gene expression [45] and stimulate an immune response against HPV, could restrain persistence of the virus and thereby the development of cervical cancer. Once the tumor is established however, TNFα could function as a tumor promoting factor. For example, TNFα promotes tumor remodeling by stimulating fibroblast and macrophage activity, tumor cell motility and invasion via the induction of matrix metalloproteinases [46-48]. In cervical cancer, Zijlmans et al. showed a positive association between *TNFα* expression and stromal TAMs [31]. These TAMs at the tumor site are thought to produce various cytokines and chemokines [49] that could promote tumor progression. *TNFα* mRNA expression in cervical cancer however was not a prognostic factor, as observed by us and Zijlmans et al. Further investigation is needed to determine whether TNFα anti-tumor or pro-tumor characteristics prevail in cervical cancer.

Transforming Growth Factor β

Transforming growth factor β (TGF-β) is a cytokine with immune and growth inhibitory properties [50,51]. Early in carcinogenesis TGF-β functions as a tumor suppressor by inhibiting cell growth whereas late in carcinogenesis TGF-β is thought to function as a tumor promoter by stimulating invasion and metastasis [52,53]. TGF-β is expressed by various tumor types including cervical cancer [54-56]. During cervical carcinogenesis, evidence exists that overexpression of TGF-β is accompanied with decreased sensitivity for the growth-limiting effect of TGF-β [57-59]. Alterations in TGF-β signaling can be caused by inactivating mutations in TGF-β signaling components. In particular inactivation of TβR-II is frequently observed in colon cancer with microsatellite instability (MSI) and inactivation of the Smad4 tumor suppressor gene in pancreatic and non MSI colon cancers [60-62]. In cervical cancer, inactivating mutations of TGF-β receptor and Smad4 have been reported mainly in cell lines at low incidence [63,64]. Alterations in TGF-β signal transduction may also be due to HPV E7 oncoproteins that bind to Smad proteins (e.g Smad2, Smad3 and Smad4) and subsequently interfere with Smad transcriptional activity by blocking binding of Smad3 protein to its DNA target sequence [65,66]. In addition, E7 may interfere with TGF-β signal transduction by blocking TGF-β suppression of c-myc transcription via pRb [67]. Inhibition of TGF-β induced suppression of c-myc transcription and inhibition of Smad DNA binding activity by E7 oncoproteins was associated with resistance to the antiproliferative effect of TGF-β [66,67]. Other mechanisms that could influence TGF-β insensitivity are defects in the TGF-β pathway downstream of Smad signaling and crosstalk of TGF-β -Smad signaling with other pathways [68,69]. TGF-β signaling can activate MAPK signaling directly, independently of Smad, and pathways like MAPK, PI3K and Wnt can influence the outcome of TGF-β signaling by crosstalk [69-71]. Many of these pathways are involved in the regulation of cell growth and cell death. This interplay of TGF-β with other pathways could enhance carcinogenesis. For example, the invasive growth of Ras-transformed epithelial cells *in vitro* depends on intact autocrine TGF-β signaling [72].

To gain insight in the resistance to TGF-β induced cell growth inhibition in cervical

cancer, we determined the effect of TGF-β on the genome-wide mRNA expression profile of cervical cancer cell lines with different sensitivity to TGF-β in **Chapter 4**. A large-scale gene expression profile was determined using cDNA microarrays of cell lines with low, intermediate and high sensitivity to TGF-β-induced growth inhibition after 0, 6, 12 and 24 hours of stimulation with TGF-β. TGF-β-altered pathways involved in cell growth regulation that affected all cell lines included Smad, MAPK, TNFα and Wnt. In all cell lines, genes that negatively regulate cell growth were induced of which many are downstream targets of Smad signaling. Only in HeLa, the cell line with high resistance to TGF-β sensitivity, genes that positively influence cell cycle progression, namely *cyclin B2* and *G2*, were upregulated. Also evidence for TGF-β-induced signaling independent of Smad was present. *MEFC2*, a target gene of p38 MAPK, was upregulated [73]. This could be mediated by TGF-β induced activation of TAK1 which is a potent activator of SAPK/JNK and p38 MAPK [74-76]. Overall, HeLa showed most of the changes in expression of genes involved in MAPK signaling. This is in line with data from Maliekal et al., who showed that reduced sensitivity of HeLa to TGF-β-induced cell growth inhibition is due to activation of MAPK [77]. In addition, TGF-β can interfere with TNFα signaling or promote TNFα induced apoptosis [78,79]. In HeLa and SiHa, cell lines with low and intermediate sensitivity to TGF-β respectively, downregulation of genes coding for receptor, adapter and signaling molecules of the TNFα pathway, partially involved in apoptosis, was observed. Downregulation of signaling components in the TNFα pathway, involved in death signaling, may interfere with TNFα induced apoptosis and possibly play a role in TGF-β resistance. Furthermore, increased gene expression in receptor, signaling and target molecules of the Wnt pathway, a pathway associated with cell growth and development, was observed especially in HeLa. This may indicate TGF-β-induced activation of this pathway. Since the pathways discussed above are involved in cell growth and cell death and have crosstalk with TGF-β signaling, they could play a role in reduced TGF-β -induced growth inhibition.

Subsequently, we investigated an association between decreased sensitivity to TGF-β-mediated cell growth inhibition and TGF-β production in the cell lines. Only the cell lines with intermediate and low sensitivity to TGF-β produced TGF-β. The cell line most resistant to TGF-β, HeLa, showed the highest concentration of TGF-β in the supernatant. Addition of anti-TGF-β did not change cell growth kinetics. Thus cell lines which are refractory to TGF-β-induced growth inhibition do excrete TGF-β in a latent form which does not affect cell growth but probably contributes to paracrine stimulation of tumor development.

TGF-β canonical pathway

In cervical cancer, some defects in the TGF-β-Smad signaling pathway have been reported [63,64] but the exact role of TGF-β-Smad signaling remains unclear. In **Chapter 5** we investigated protein expression of Smad2, Smad2P, Smad4 and p21, a major target of TGF-β-Smad signaling [80], in a large group of cervical carcinomas. All cervical carcinomas, assembled in a tissue array (n = 118) expressed Smad2, Smad2P and Smad4, as determined by immunohistochemistry. Also nuclear staining

of p21 was observed in the majority of tumors (n = 99). These data suggest a functional TGF-β-Smad signaling pathway in most cervical carcinomas. Nonetheless, cytoplasmic staining was weak for Smad2 in 33% and for Smad4 in 36% of tumors. To establish the basis for the weak Smad2 and Smad4 protein expression, we have investigated whether this was due to haplo-insufficiency of the Smad genes caused by loss of heterozygosity (LOH) or inactivating mutations. From the 46 cases analyzed, 40% showed loss at the locus of *Smad2* and *Smad4* which did not associate with weak Smad2 or Smad4 protein expression. Furthermore, no mutations in the functional MH1 and MH2 domains of *Smad2* or *Smad4* were observed in tumors with weak expression and/or absent nuclear staining.

In cancer, most mutations of *Smad2* and *Smad4* occur in the MH1 and MH2 domain, thereby affecting phosphorylation, nuclear translocation or protein stability [81,82]. One other study investigated *Smad2* and *Smad4* mutations in cervical carcinomas and reported one genetic aberration; an insertion in the MH2 domain of *Smad2* in 1 tumor, resulting in loss of expression [64]. No genetic alterations were found of *Smad4* in 20 tumors investigated [64]. These and our data suggest that genetic mechanisms for downregulation of Smad2 and Smad4 expression in cervical carcinomas are uncommon.

An epigenetic mechanism for downregulation of expression, methylation of CpG islands in the promoter region of *Smad4* was studied in cervical cancer cell lines, normal samples and tumor samples. None of the CpG sites investigated were methylated. A few studies investigated CpG methylation of the *Smad4* promoter as a mechanism for downregulation of expression in cancer [83-86]. These studies investigated different but overlapping areas of the promoter and the data obtained was not conclusive. Perhaps other epigenetic mechanisms, possibly in addition to methylation, such as histone acetylation and other chromatin remodeling machinery, may be required.

Furthermore, we investigated whether the expression pattern of Smad2, Smad2P and Smad4 associated with clinico-pathological parameters and survival. Smad4 weak cytoplasmic staining and lack of Smad4 nuclear staining correlated with tumor size and poor survival. In other tumors such as pancreatic and colorectal cancer, absent Smad4 protein expression was shown to be an unfavorable prognostic factor [87,88]. Low presence of Smad4 in the nucleus (and cytoplasm) of cervical tumors could mean that Smad4 target genes are insufficiently induced upon TGF-β signaling. Some of these target genes, such as p15 and p21, are involved in cell growth inhibition [51]. Surprisingly, Baldus et al. reported that all cervical cancer cell lines analyzed showed either absent or moderate responsiveness to TGF-β irrespective of their Smad4 status [63]. This has also been reported for colorectal and pancreatic cancer cell lines [89,90]. In addition to growth regulation, other tumor suppressing properties have been attributed to Smad4. Schwarte-Waldhoff et al. showed that the tumor-suppressive role of Smad4, *in vivo*, associated with morphological changes and improved adhesive properties of tumor cells [89]. This was accompanied with a reduced expression level of uPA and PAI-1, proteins that are implicated in the control of cell adhesion and invasion [19]. Smad4 was also shown to inhibit angiogenesis *in vivo* by reducing the expression of an important angiogenesis inducer, vascular endothelial

growth factor, and inducing the angiogenesis inhibitor thrombospondin 1 [90]. Thus besides being involved in growth inhibition, Smad4 exhibits other tumor suppressive properties which may be more important in cancer because often tumor cells acquire resistance to growth inhibition due to constantly active proliferating pathways like Erk MAPK [91].

Overall, Chapter 4 and 5 provide evidence that the canonical TGF-β-Smad pathway in cervical cancer does not seem to harbor major deficits (e.g. mutations in *Smad2* and *Smad4*) but suggests that one of the contributory causes for alterations in signal transduction is crosstalk with other pathways and/or direct TGF-β-induced stimulation of these pathways, thereby altering/overruling signaling via Smads.

Genome-wide molecular alterations in cervical cancer

Host genomic alterations in addition to HPV infection are needed for precursor lesions to progress to invasive cancer. The accumulation of these genomic alterations is enhanced by the increased genomic instability that occurs as a result of high E6 and E7 expression after viral integration [92]. These include aberrations that affect tumor suppressor genes or oncogenes critical for tumor development. Multiple genetic alterations have been found in cervical cancer mainly by methods like metaphase comparative genomic hybridization (mCGH) and microsatellite marker analysis for the detection of LOH [93]. These techniques have resulted in the discovery of only a few genes, targeted by genomic imbalances and possibly involved in carcinogenesis, due to the lack of resolution and laborious work involved. Nowadays, techniques that allow detailed genome-wide analyses have become available, including array comparative genomic hybridization (array CGH), single nucleotide polymorphism array (SNP array) and gene expression arrays.

In **Chapter 6**, the genomes of 10 cervical cancer cell lines were examined in order to evaluate common regions of gains and losses and to identify genes involved in cervical carcinogenesis, particularly in immune response. Complex large-scale genetic changes were observed in the cancer cell lines. Many of these genetic alterations such as gain of 5p and 20q and loss of 4p and 13q, have been reported frequently in cervical cancer [94-96]. Most chromosomes were affected by LOH. In the cell lines, chromosome arm 6p belonged to those regions targeted most frequently by LOH, which has been reported previously [97]. A number of genes involved in immune regulation and antigen presentation are located at 6p including TNFα, HLA and peptide transporter TAP. The tumor probably benefits from loss of 6p since it may facilitate evasion of anti-tumor immune responses.

Genome-wide, no correlation between copy-number changes and mRNA expression were observed, suggesting that other mechanisms for transcriptional regulation are probably also important. Amplification of 5p though, correlated with a significant higher expression for 22% of the genes. Genes at 5p, such as *SKP2*, *TRIO* and *ANKH*, have been previously reported in association with other cancers [98-100]. Besides amplifications of these genes in different types of neoplasms, SKP2 and TRIO were shown to promote tumor cell proliferation [100,101]. Recently it has been shown that SKP2 is involved in TGF-β signal transduction. Upon TGF-β

stimulation, SKP2 is rapidly degraded, through signaling by the canonical Smad pathway, which results in stabilization of the cell cycle arrest protein p27, thereby ensuring TGF-β -mediated cell cycle arrest [102]. However, in a recent paper that investigated p27 and SKP2 protein expression in a large series of cervical carcinomas (n = 332) no correlation between p27 and SKP2 expression was found. Tumor stage was the only parameter with prognostic significance in case of SKP2 expression [103]. Future research has to reveal whether this oncogene is involved in cervical carcinogenesis for instance by investigating its effect on growth inhibition upon siRNA mediated knock down of *SKP2* in cervical cancer cells.

Conclusions

The studies described in this thesis focus on molecular aspects of immune escape mechanisms in addition to HLA class I loss, including loss of TNFα expression and TGF-β insensitivity in cervical cancer (Figure 1). Tumors with partial and normal HLA expression as compared to tumors negative for HLA class I showed an overall higher expression profile of genes involved in proinflammatory responses such as the chemokines *CCL3* and *CCL4* and acute phase proteins *SerpinA1* and *SerpinA3*. We identified *SerpinA1* and *SerpinA3* as candidate genes involved in immune suppression and/or carcinogenesis in HLA positive tumors. Besides HLA class I loss, we observed that loss of TNFα expression is a frequent event in cervical cancer. Based on our findings we can conclude that genetic inactivation of TNFα as a cause for lack of expression of TNFα is unlikely. The epigenetic regulation of TNFα expression and the functional significance in cervical cancer remain to be elucidated. Using an *in vitro* model, we have shown that TGF-β insensitivity in cervical cancer cells is associated with activation of pathways involved in proliferation like MAPK. Therefore, we speculate that TGF-β insensitivity in cervical cancer is caused by changes in signal transduction due to crosstalk of the TGF-β-Smad pathways with these pathways and/or TGF-β -induced stimulation of these pathways. In accordance with this view, we provide evidence by means of an immunohistochemical study of Smad protein expression that the TGF-β-Smad signaling pathway is functional in most cervical carcinomas and that inactivation of *Smad2* or *Smad4* genes as a cause for TGF-β insensitivity is unlikely. Finally, we aimed at identifying genome-wide alterations and genes involved in cervical carcinogenesis. A genome-wide analysis of cervical cancer cell lines on the DNA and RNA level indicated 3 candidate genes, *SKP2*, *TRIO* and *ANKH* at 5p that might be involved in the pathogenesis of cervical cancer. In conclusion, our studies provide evidence that cervical cancer cells develop various strategies to evade eradication by immune cells. These include loss of HLA class I, lack of TNFα expression, resistance to TGF-β growth inhibition, production of TGF-β and possibly SerpinA1 and/or SerpinA3 expression. Therefore, when treating cervical cancer patients with immunotherapy, one should take in account the array of escape tactics that cervical cancer cells employ.

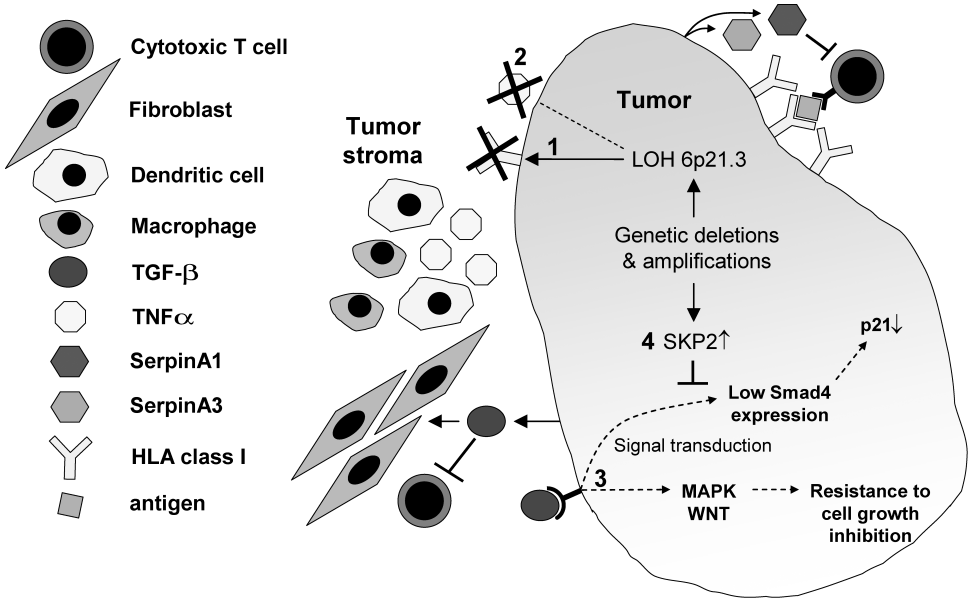


Figure 1. Investigated immune escape mechanisms. 1. Cervical tumors show frequent loss of HLA class I expression. *Immune escape routes in HLA class I positive tumors* were investigated. HLA class I positive tumors showed strong expression of SerpinA1/SerpinA3 as a possible strategy of the tumor to escape immune surveillance (chapter 2). A common genetic alteration resulting in loss of HLA class I is loss of heterozygosity (LOH) at 6p21.3, the locus of HLA class I and TNFα. 2. *Loss of TNFα expression* due to LOH, mutation and/or promoter methylation was investigated. Genetic inactivation as a cause for lack of TNFα expression is unlikely (chapter 3). 3. *Loss of TGF-β sensitivity*. Resistance to TGF-β-induced growth inhibition is accompanied with stimulation of growth promoting pathways (MAPK and WNT) that could possibly counteract or overrule the growth inhibiting signal of TGF-β via Smads (chapter 4). Low cytoplasmic and absent nuclear staining of Smad4, a member of the canonical TGF-β pathway, may influence cervical carcinogenesis since it correlated with poor outcome (chapter 5), emphasizing the importance of the integrity of the canonical TGF-β pathway. 4. Performing genome-wide analysis (chapter 6), we observed *amplification and overexpression of SKP2*, a candidate oncogene which plays a role in TGF-β signaling since it needs to be degraded in order to accomplish TGF-β mediated cell cycle arrest.

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8

Nederlandse Samenvatting

Wereldwijd bezien hoort baarmoederhalskanker tot de tweede meest voorkomende kanker bij vrouwen. Het merendeel komt voor in ontwikkelingslanden, onder meer vanwege een gebrek aan bevolkingsonderzoeken die opsporing en behandeling van (pre)maligne afwijkingen mogelijk maken. De tumor ontwikkelt zich in de transformatiezone, de overgang van plaveiselepitheel aan de buitenkant naar cilindrisch epitheel aan de binnenkant, van de baarmoederhals. De ontwikkeling tot baarmoederhalskanker verloopt via verschillende voorstadia genaamd Cervicale Intraepitheliale Neoplasie (CIN)1, CIN2 en CIN3, die opeenvolgend toenemen in mate van dysplasie. Voortdurende infectie met een hoog-risico type humaan papilloma virus (HPV) speelt een belangrijke rol in de ontwikkeling van baarmoederhalskanker. Een tweede belangrijke factor betreft genetische veranderingen die optreden in de cel, mede veroorzaakt door genomische instabiliteit na integratie van het HPV DNA in het genoom van de gastheercel. Ten derde is de immuunrespons van belang. Met name de cellulaire afweer is belangrijk bij de genezing van een HPV infectie en de regressie van (pre)maligne afwijkingen. De beschreven studies in dit proefschrift betreft onderzoek naar verschillende ontsnappingsroutes van baarmoederhalskankercellen aan eliminatie door het immuunsysteem.

Een veelvoorkomend mechanisme van baarmoederhalskankercellen om te ontsnappen aan eliminatie door het immuunsysteem is verlies van het zogenaamde humaan leucocyte antigeen (HLA) klasse I. Met behulp van deze HLA eiwitten worden zowel lichaamseigen als lichaamsvreemde stukjes eiwit, peptiden genaamd, aan het immuunsysteem gepresenteerd. Door verlies van DNA dat codeert voor HLA klasse I in de tumorcel voorkomt deze dat virale en tumorspecifieke peptiden worden gepresenteerd en blijft de tumorcel onzichtbaar voor het immuunsysteem.

Om inzicht te krijgen in immuunontsnappingsroutes van tumoren zonder HLA verlies werden in **Hoofdstuk 2** tumoren met en zonder verlies van HLA klasse I met elkaar vergeleken. De HLA status van tumoren werd bepaald op eiwit niveau met behulp van immunohistochemie, gebruikmakend van antilichamen tegen β2-microglobuline, HLA-A en HLA-B/C, en op genetisch niveau door het bepalen van HLA klasse I haplotype verlies. Dit laatste werd gedaan door het bepalen van verlies van heterozygoziteit (LOH) van de HLA regio op 6p21.3 met behulp van markers. Een genoomdekkend genexpressie-profiel van tumoren met volledig, gedeeltelijk (LOH 6p21.3) en geen/minimaal HLA klasse I (HLA negatieve tumoren) verlies werd bepaald d.m.v. microarray analyse. Daaruit bleek dat het expressie-profiel van tumoren met gedeeltelijk en geen/minimaal verlies van HLA klasse I (HLA positieve tumoren) sterk overeenkwam en een duidelijk verhoogde expressie van “immuunrespons” genen toonde in vergelijking met HLA negatieve tumoren. Met name de categoriën “Ontsteking” en “Acute fase respons” verschilden tussen de HLA negatieve en HLA positieve tumoren. Binnen deze categoriën toonden de acute fase eiwitten *SerpinA1* en *SerpinA3* een duidelijk hogere genexpressie in HLA positieve tumoren. Dit verschil in mRNA expressie werd bevestigd m.b.v. real-time PCR, een semi-kwantitatieve methode om mRNA te meten, en op eiwit niveau m.b.v. immunohistochemie. Een hoge expressie van SerpinA1 of SerpinA3 bij patiënten met HLA positieve tumoren correleerde met een verkorte overlevingsperiode. Eerder gepubliceerd onderzoek

toonde een immuunsuppressieve rol aan voor SerpinA1 middels het remmen van de cytotoxociteit van NK-cellen en een apoptose-remmende werking van SerpinA3. Dit suggereert dat expressie van deze eiwitten in tumoren een rol zou kunnen spelen in ontsnapping aan het immuunsysteem. Toekomstig onderzoek zal moeten bepalen op welke wijze SerpinA1 en/of SerpinA3 expressie in baarmoederhalskanker bijdraagt aan het ontsnappen aan de afweer.

Cytokines zijn signaalstoffen die betrokken zijn bij de regulatie van de immuunrespons. Cytokines kunnen ingedeeld worden in ontstekings-remmende en ontstekings-stimulerende eiwitten. Tumor necrosis factor α (TNFα) is een cytokine behorend tot de laatste categorie en speelt mogelijk een rol in het doden van tumorcellen door geactiveerde macrofagen en/of het stimuleren van een cellulaire immuunrespons. De afwezigheid van immuunstimulerende cytokines, zoals TNFα, zou een mechanisme kunnen zijn om aan het immuunsysteem te ontsnappen.

In **Hoofdstuk 3** is onderzoek naar de expressie van *TNFα* in baarmoederhalskanker beschreven. Tevens zijn (epi)genetische mechanismen bestudeerd in geval van lage of afwezige *TNFα* expressie. In baarmoederhalkanker gaat een regio van chromosoomarm 6p waar het *TNFα* gen ligt, evenals de *HLA* regio, verloren in de helft van de tumoren. *TNFα* mRNA expressie, aangetoond m.b.v. RNA *in situ* hybridisatie, was in 45% van de onderzochte tumoren niet aantoonbaar in de maligne cellen. Er waren geen aanwijzingen voor genetische afwijkingen van het *TNFα* gen, zoals mutatie, deletie of LOH van 6p21.3 waarmee verlaagde expressie of afwezigheid van *TNFα* expressie verklaard kon worden. Tevens bleek het TNF2 allel, dat geassocieerd is met hogere TNFα expressie, geen specifiek doelwit te zijn voor verlies d.m.v. LOH. Behalve een genetisch defect zou een epigenetisch mechanisme zoals CpG methylering van de *TNFα* promoter de expressie kunnen verminderen. CpG methylering van de *TNFα* promoter als epigenetisch mechanisme lijkt onwaarschijnlijk omdat de meeste tumoren zonder *TNFα* expressie geen CpG methylering van de promoter en exon 1 hadden. Waarschijnlijk is een ander, nog niet geïdentificeerd, mechanisme actief dat *TNFα* expressie verhinderd.

TGF-β is een cytokine met verscheidene immuunsuppressieve eigenschappen waaronder het remmen van T-cel proliferatie en differentiatie, welke cruciale processen zijn in de cellulaire afweer tegen tumorcellen. Daarnaast remt TGF-β de groei van o.a. epitheliale cellen. De TGF-β signaaltransductie, die onder meer leidt tot remming van de celgroei, verloopt via Smad eiwitten en resulteert in de expressie van cycline afhankelijke kinase remmers zoals p15 en p21 die de celcyclus remmen. De rol van TGF-β in carcinogenese is complex. Vroeg in de carcinogenese speelt TGF-β een rol als tumorsuppressor, door het remmen van celgroei, terwijl er aanwijzingen zijn dat TGF-β laat in de carcinogenese kanker bevorderd door het stimuleren van invasieve groei en metastasering. Gedurende de ontwikkeling van baarmoederhalskanker lijkt er een afname te zijn van het groeiremmende effect van TGF-β.

Om inzicht te krijgen in de ongevoeligheid van tumorcellen voor TGF-β is het genexpressie-profiel onderzocht van 3 cellijnen met een verschillende gevoeligheid voor TGF-β m.b.v. micorarray in **Hoofdstuk 4**. Alle 3 de cellijnen toonden een

verhoogde expressie van genen die doelwit zijn van de TGF-β-Smad signal route na stimulatie met TGF-β. Ook werden in de cellijnen veranderingen in expressie waargenomen van signaalroutes betrokken bij celgroei en celdood zoals MAPK, Wnt en TNFα. Het is bekend dat deze signaalroutes en de TGF-β-Smad signaalroute elkaar kunnen beïnvloeden. De genexpressie veranderingen in de TGF-β ongevoelige cellijn duiden op activatie van de MAPK (p38 en ERK) en de Wnt signaalroute, beiden betrokken bij celgroei. Dit zou een rol kunnen spelen in de ongevoeligheid van de tumorcel voor TGF-β doordat het groeiremmende signaal via Smad eiwitten mogelijk onderdrukt wordt door het groei-stimulerende signaal via MAPK en/of Wnt. Tevens is onderzocht of de cellijnen zelf TGF-β produceren. Alleen de 2 cellijnen die (deels) ongevoelig waren voor TGF-β produceerden TGF-β eiwit. Productie van 'eigen' TGF-β had geen effect op de celgroei. Dus alleen cellijnen met enige ongevoeligheid voor TGF-α produceerden TGF-β in een latente vorm. Dit TGF-β draagt waarschijnlijk bij aan de paracrine stimulatie van tumorcelgroei.

Een verstoring in de TGF-β-Smad signaal route zou eveneens een rol kunnen spelen in de carcinogenese van baarmoederhalskanker. Mutaties in het gen dat codeert voor Smad4 zijn aangetroffen in baarmoederhalskanker. In kanker van de alvleesklier staat *Smad4* bekend als 'tumorsuppressorgen' waarbij het gen vaak geïnactiveerd wordt d.m.v. LOH in combinatie met mutatie. Ook mutaties in het *Smad2* gen zijn aangetroffen in enkele tumoren.

In **Hoofdstuk 5** is onderzoek naar de eiwitexpressie, bepaald m.b.v. immunohistochemie, van Smad2, Smad4 en p21, een eindproduct van de TGF-β-Smad signaal route, in 118 tumoren beschreven. Alle tumoren waren positief voor Smad2, P-Smad2, de gefosforyleerde vorm van Smad2, wat duidt op activatie van de Smad signaal route, en Smad4. De meeste tumoren waren positief voor p21 (n = 99). Deze resultaten wijzen op een functionele TGF-β-Smad signaal route in de meeste tumoren. Een derde van de tumoren had een zwakke expressie van Smad2 of Smad4. Vervolgens is onderzocht of een (epi)genetisch defect een verklaring hiervoor zou kunnen zijn. LOH van 18q21, de locus van *Smad2* en *Smad4*, werd aangetroffen in 40% van de tumoren maar toonde geen verband met lage expressie. Er werden geen afwijkingen gevonden in het gen dat codeert voor *Smad2* en *Smad4* en CpG methylering van de *Smad4* promoter werd niet gedetecteerd. Deze bevindingen tonen aan dat inactivatie van de genen die coderen voor *Smad2* en *Smad4* geen veelvoorkomend verschijnsel is in baarmoederhalskanker. Tevens is de relatie van Smad expressie met klinisch-pathologische parameters onderzocht. Zwakke cytoplasmatische expressie en afwezige kernkleuring van Smad4 toonde een relatie met tumorgrootte en slechtere overleving. Een aannemelijke verklaring is dat geen of te weinig Smad4 eiwit in de kern, waar Smad4 als transcriptiefactor functioneert, de expressie van zogenaamde 'target'-genen onvoldoende stimuleert. Deze genen waaronder *p15*, *p21* en *p27*, zijn betrokken bij de regulatie van de celcyclus en oefenen een remmende werking uit op celgroei. Echter i.t.t. wat men zou verwachten zijn cellijnen met verlies van Smad4 niet minder gevoelig voor TGF-β. Wel zijn er andere tumorremmende eigenschappen toegeschreven aan Smad4. Zo is aangetoond dat Smad4 de adhesie van tumorcellen bevordert en angiogenese remt.

Uit Hoofdstuk 4 en 5 kan geconcludeerd worden dat de TGF-β-Smad signaal route in baarmoederhalskanker geen grote gebreken vertoond maar dat TGF-β stimulatie leidt tot activatie van signaal routes betrokken bij celgroei en celdood die mogelijk een rol zouden kunnen spelen in ongevoeligheid van tumorcellen voor TGF-β-geïnitieerde celgroeiremming.

Voor de ontwikkeling van baarmoederhalskanker zijn naast een voortdurende infectie met HPV ook genetische veranderingen van cruciaal belang. De opeenhoping van genetische veranderingen wordt bevorderd door de genomische instabiliteit welke veroorzaakt wordt door de expressie van de virale eiwitten E6 en E7. Deze genetische veranderingen treffen ook 'tumorsuppressorgenen' en 'oncogenen'. Verlies van genetisch materiaal d.m.v. LOH kan wijzen op de mogelijke aanwezigheid van een tumorsuppressorgen. Dit kunnen genen zijn die een belangrijke rol spelen in de immuunrespons tegen HPV. Een bekend voorbeeld is LOH van 6p21.3 waardoor er verlies van HLA klasse I allelen plaatsvindt in de helft van de baarmoederhalstumoren. Tegenwoordig zijn er technieken beschikbaar waarmee tot in detail verlies en winst van genetisch materiaal over het hele genoom kan worden gemeten zoals array CGH en SNP array.

Met behulp van deze technieken hebben wij in **Hoofdstuk 6** het genoom van 10 baarmoederhalskankercellijnen bestudeerd om veelvoorkomende gebieden van verlies en winst en genen betrokken bij carcinogenese, met name bij de immuunrespons, te identificeren. Complexe en grootschalige genetische veranderingen waarbij complete chromosoomarmen betrokken zijn, werden geobserveerd in de cellijnen. Veel van deze genetische veranderingen zoals winst van chromosoomarm 5p en 20q en verlies van 8p, 4p en 13q, zijn eerder beschreven in baarmoederhalskanker. Ook toonden de meeste cellijnen LOH van 6p, een regio van het genoom waar zich veel genen bevinden die een rol spelen in de immuunrespons zoals HLA klasse I. Daarnaast is de genexpressie m.b.v. microarray in de cellijnen bestudeerd en zijn de data gecombineerd met de array CGH resultaten om te bepalen of het niveau van genexpressie verandert met het verlies of winst van genetisch materiaal. Op chromosoomarm 5p kwamen 22% van de genen hoger tot expressie in de cellijnen die winst toonden van 5p. Voor 3 van deze genen op 5p, *SKP2*, *TRIO* en *ANKH*, is deze hogere genexpressie bevestigd m.b.v. 'real-time PCR'. Deze genen zijn eerder met kanker in verband gebracht en aangetoond is dat SKP2 en TRIO tumorcelgroei bevorderen. Recent is ook een publicatie verschenen over de rol van SKP2 in de TGF-β signaal route. Stimulatie van de TGF-β-Smad signaal route heeft tot gevolg dat SKP2 snel afgebroken wordt waardoor stabilisatie van p27 plaatsvindt en de celcyclus geremd wordt. Mogelijk zou SKP2 overexpressie een rol kunnen spelen in de ongevoeligheid van tumorcellen voor TGF-β-geïnitieerde celgroeiremming. Er kan geconcludeerd worden uit deze studie dat baarmoederhals-tumorcellen zeer complexe genetische veranderingen ondergaan en dat *SKP2*, *TRIO* en *ANKH* mogelijk doelwit zijn voor de aangetoonde winst op chromosoomarm 5p.

Conclusies

Het doel van dit proefschrift was het onderzoeken van immuunontsnappingsmechanismen van tumorcellen bij patiënten met baarmoederhalskanker. Allereerst werd onderzocht wat voor mechanismen bij HLA positieve tumoren voorkomen. *SerpinA1* en *SerpinA3* zijn geïdentificeerd als mogelijke genen betrokken bij het onderdrukken van de afweer in HLA positieve tumoren. Ten tweede kan het niet tot expressie brengen van immuunstimulerende cytokines als TNFα resulteren in het ontsnappen van tumorcellen aan de afweer. Geconcludeerd kan worden dat ongeveer de helft van de tumoren geen TNFα tot expressie brengt en dat hierbij inactivatie van het gen coderend voor TNFα onwaarschijnlijk is. Een derde mechanisme is ongevoeligheid voor het groeiremmende effect van TGF-β. Uit onze resultaten blijkt dat TGF-β ongevoeligheid gepaard gaat met activatie van signaal routes betrokken bij celgroei zoals MAPK. Dus TGF-β ongevoeligheid voor groeiremming zou het gevolg kunnen zijn van TGF-β stimulatie van signaal routes die celgroei stimuleren naast de signaal overdracht via Smad eiwitten die eveneens celgroei reguleren, waarbij de eerstgenoemde de overhand krijgt. Ontregeling van de TGF-β -Smad signaal route door inactivatie van *Smad2* of *Smad4* zou eveneens een rol kunnen spelen bij het ontstaan van baarmoederhalskanker. Onze studie betreffende eiwitexpressie van Smad2 en Smad4 impliceert echter dat de TGF-β -Smad signaal route functioneel is in de meeste tumoren. De laatste studie beschrijft een uitgebreide genomische analyse waarbij 3 genen op 5p geïdentificeerd werden die mogelijk een rol spelen bij de carcinogenese van baarmoederhalskanker: *SKP2*, *ANKH* en *TRIO*. Concluderend impliceren de gegevens uit dit proefschrift dat in baarmoederhalskanker verscheidene mechanismen een rol spelen die bijdragen aan het ontsnappen aan het immuunstysteem. De bevindingen zijn in een model samengevat, weergegeven in Figuur 1 (Hoofdstuk 7, blz 126).

Curriculum Vitae

Judith Kloth werd geboren op 14 juni 1974 in Delft. In 1991 behaalde zij het HAVO diploma aan het Gertrudiscollege te Roosendaal. In 1992 werd begonnen met de Hogere Laboratorium Opleiding (HLO) te Etten-Leur. De afstudeerstage in 1996 betrof onderzoek aan het proto-oncogen c-Src, welke plaatsvond in het Friedrich Miescher Instituut in Basel te Zwitserland, onder begeleding van Dr. K. Balmer. Het HLO examen werd behaald in juni 1996. Van augustus 1996 tot juli 1998 was zij werkzaam als analist in de onderzoeksgroep 'Chirurgische Oncologie' welke geleid werd door Prof. Dr. M. Heberer en Prof. Dr. G.C. Spagnoli bij het 'Zentrum für Lehre und Forschung' van 'Kantonsspital' Basel. In september 1998 werd aangevangen met de verkorte studie 'Medische Biologie' aan de Vrije Universiteit in Amsterdam. In januari 2000 liep zij stage op de medische afdeling van het pharmaceutisch bedrijf Eli Lilly in Nieuwegein onder begeleiding van Drs. R. Kroes en in september begon ze haar sciptie over gentherapie bij kanker bij 'New Drug Development Office Oncology' te Amsterdam onder begeleiding van Dr. M. Krul. Het doctoraal examen werd behaald in februari 2001. In januari 2002 werd aangevangen met het promotie-onderzoek in het LUMC te Leiden bij de vakgroep Pathologie in de onderzoeksgroep Tumorimmunologie onder leiding van Prof. Dr. G.J. Fleuren, Dr. A. Gorter en Prof. Dr. G.G. Kenter. Eind 2007-begin 2008 werkte zij als onderzoekscoördinator voor 4 maanden bij KWF Kankerbestrijding. Sinds juni 2008 werkt zij als medisch beoordelaar bij het College ter Beoordeling van Geneesmiddelen.

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Kloth JN, Kenter GG, H. Spijker S, Uljee S, Corver WE, Jordanova ES, Fleuren GJ and Gorter A. Expression of Smad2 and Smad4 in cervical cancer: absent nuclear Smad4 expression correlates with poor survival. *Modern Pathology*. 2008; 21(7): 866-875.

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Piersma SJ, Welters MJ, van der Hulst JM, *Kloth JN*, Kwappenberg KM, Trimbos BJ, Melief CJ, Hellebrekers BW, Fleuren GJ. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. *International Journal of Cancer*. 2008; 122(3): 486-494.

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Dankwoord

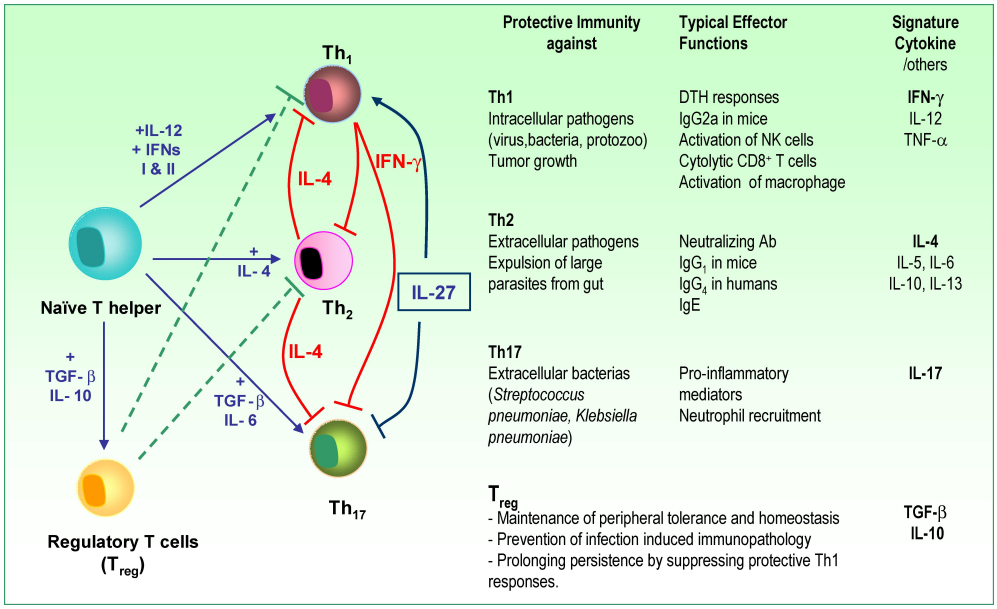
Bij deze wil ik de mensen bedanken die bijgedragen hebben aan de totstandkoming van dit proefschrift.

Allereerst wil ik iedereen op de Pathologie bedanken voor de prettige werksfeer, behulpzaamheid en adviezen. De ‘thee’-pauzes, labuitjes en borrels waren een welkome afleiding tijdens het promotie-onderzoek. Mijn kamergenoten wil ik bedanken voor het delen van kennis en de leuke contacten. Speciaal wil ik de volgende personen bedanken voor hun bijdrage aan dit proefschrift: Katja Jordanova, Jan Oosting, Natalja ter Haar, Sandra Kolkman-Uljee, Siebe Spijker, Wim Corver, Jaap Eendenburg, Hans Baelde, Henry Zijlmans, Brendy van den Akker, Enno Dreef, Karoly Szuhai, Tom van Wezel, Anne-Marie Cleton, Cees Cornelisse en Klaas van der Ham, voor de samenwerking, technische ondersteuning en hulp met statistiek en photoshop.

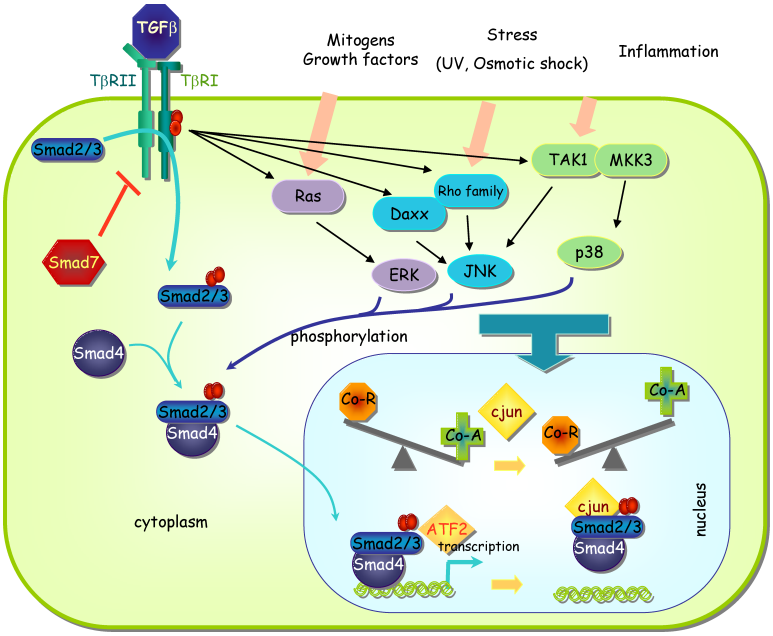
Mijn ouders, zussen en vrienden wil ik bedanken voor hun steun, interesse en een leuke tijd naast het onderzoek.

Tenslotte dank ik John. Bij jou vond ik rust en relativering op momenten dat ik het goed kon gebruiken. Je morele steun heeft substantieel bijgedragen aan het tot een goed einde brengen van het promotieonderzoek!

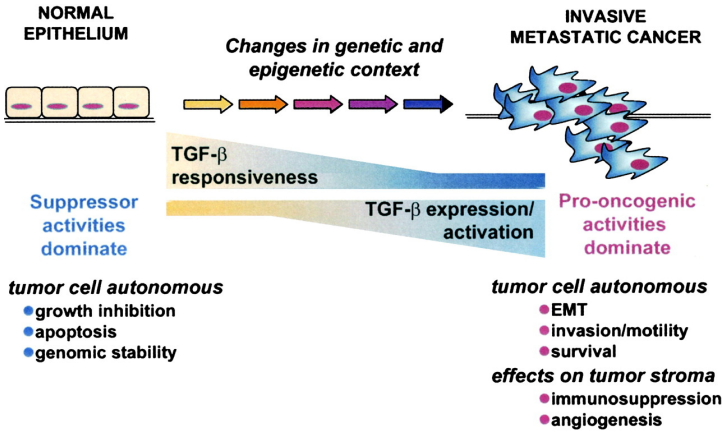
Appendix: Colour Illustrations



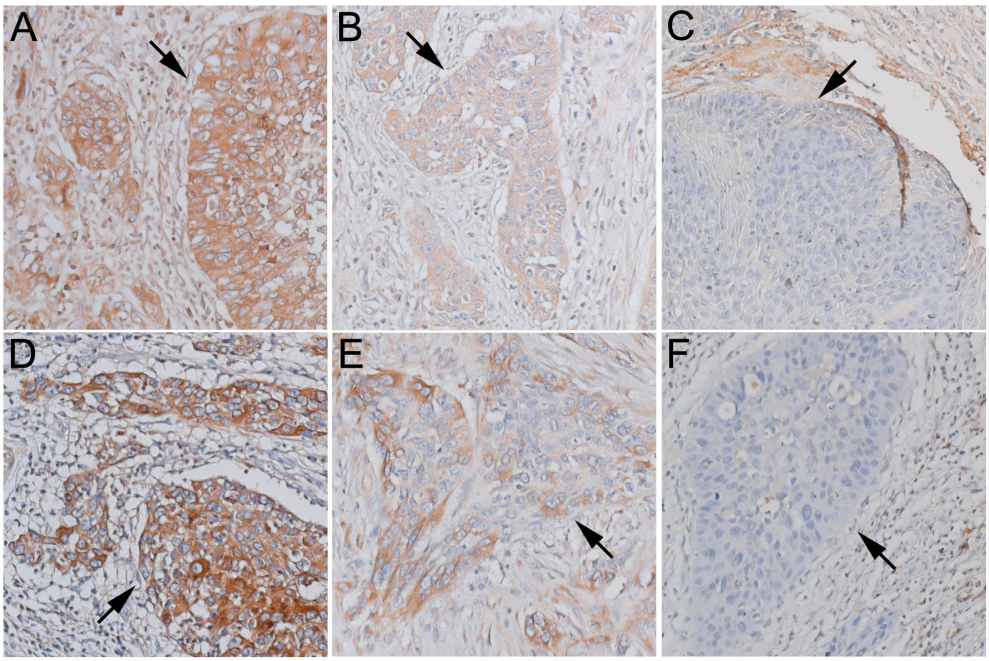
Chapter 1, Figure 1. Cytokines involved in differentiation of helper T lymphocyte subsets. Abbreviations: DTH, delayed type hypersensitivity; Ab, antibodies. From Chabalgoity et al., 2007.



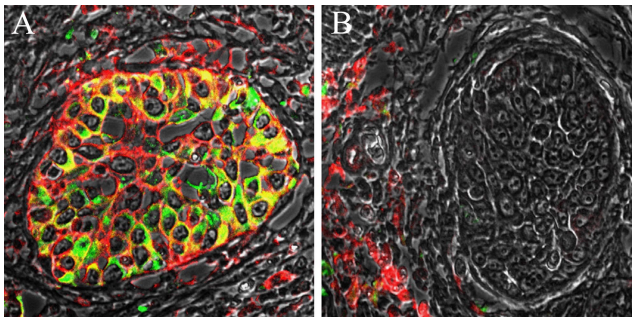
Chapter 1, Figure 2. TGF-β induced activation of Smad and MAPK and their interactions. Activation of Smad signaling occurs once TGF-β binds to the TGF-β receptor type II (TβRII). Subsequently, TβRII phosphorylates TGF-β receptor type I (TβRI) which in turn phosphorylates Smad2/3. Then, activated Smad2/Smad3 bind Smad4 and translocate to the nucleus where the complexes function as transcription factors. Transcription is controlled by the presence of coactivators (Co-A) or corepressors (Co-R). Activation of the 3 MAPK (ERK, JNK and p38) by TGF-β or other stimuli such as growth factors or proinflammatory cytokines, can regulate Smad activation by direct phosphorylation or through downstream effector molecules. Examples of downstream effector molecules are cjun and ATF2 which modulate transcriptional activity. From Javelaud et al., 2005.



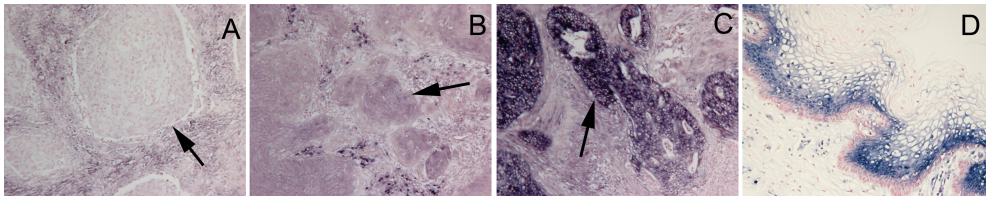
Chapter 1, Figure 3. TGF-β switches from tumor suppressor in normal and premalignant stages of tumorigenesis to proto-oncogene at later stages of disease. Progression to metastatic disease is usually accompanied by decreased or altered TGF-β responsiveness and increased expression/activation of TGF-β. Besides changes in the responsiveness to TGF-β in tumor cells, TGF-β effects tumor stroma in such a manner that tumor growth and metastasis is facilitated. From Roberts et al., 2003.



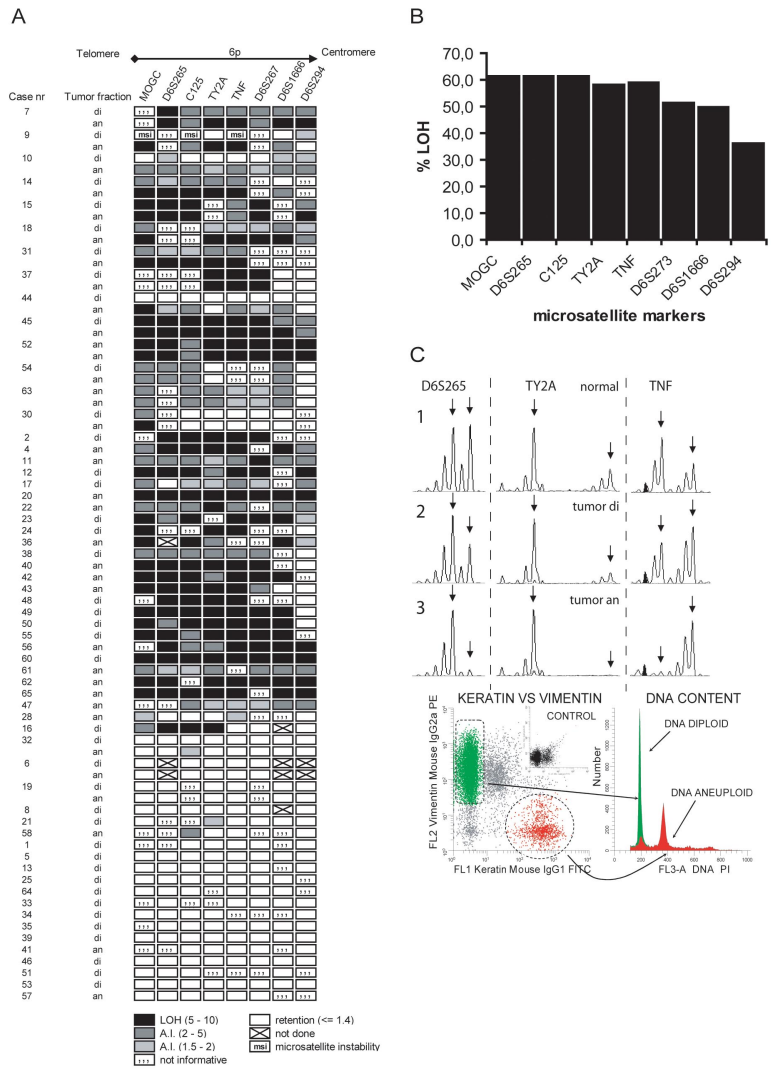
Chapter 2, Figure 4. Immunohistochemical analysis of SerpinA1 and SerpinA3 protein expression in cervical carcinomas. Arrows indicate tumor areas. A) Strong staining of SerpinA1, B) weak staining of SerpinA1, C) lack of SerpinA1 staining, D) strong staining of SerpinA3, E) mostly weak staining of SerpinA3 and F) lack of SerpinA3 staining.



Chapter 2, Figure 5. Immunofluorescent staining for SerpinA3 and HLA class B/C. Tumors are depicted with A) positive staining for HLA class I (red, membranous) and SerpinA3 (green, cytoplasmic) or B) no positive staining for HLA class I and SerpinA3, whereas infiltrate cells show positivity.

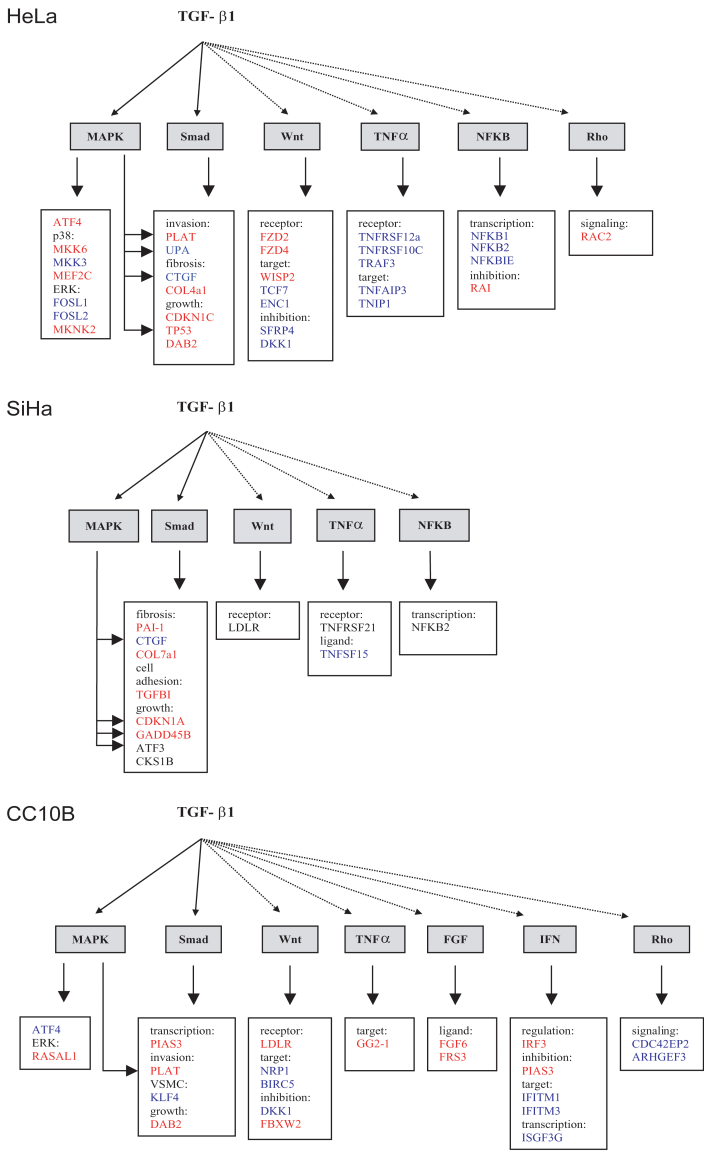


Chapter 3, Figure 1. TNFα mRNA expression in cervical cancer and normal cervical squamous epithelium (x 100). mRNA expression was measured by RISH as described in Materials and methods. The arrows point to tumor areas. A) Squamous cell carcinoma showing no TNFα expression, B) Squamous cell carcinoma showing low TNFα expression, C) Adenocarcinoma showing high TNFα expression and D) Normal cervical squamous epithelium showing TNFα expression.

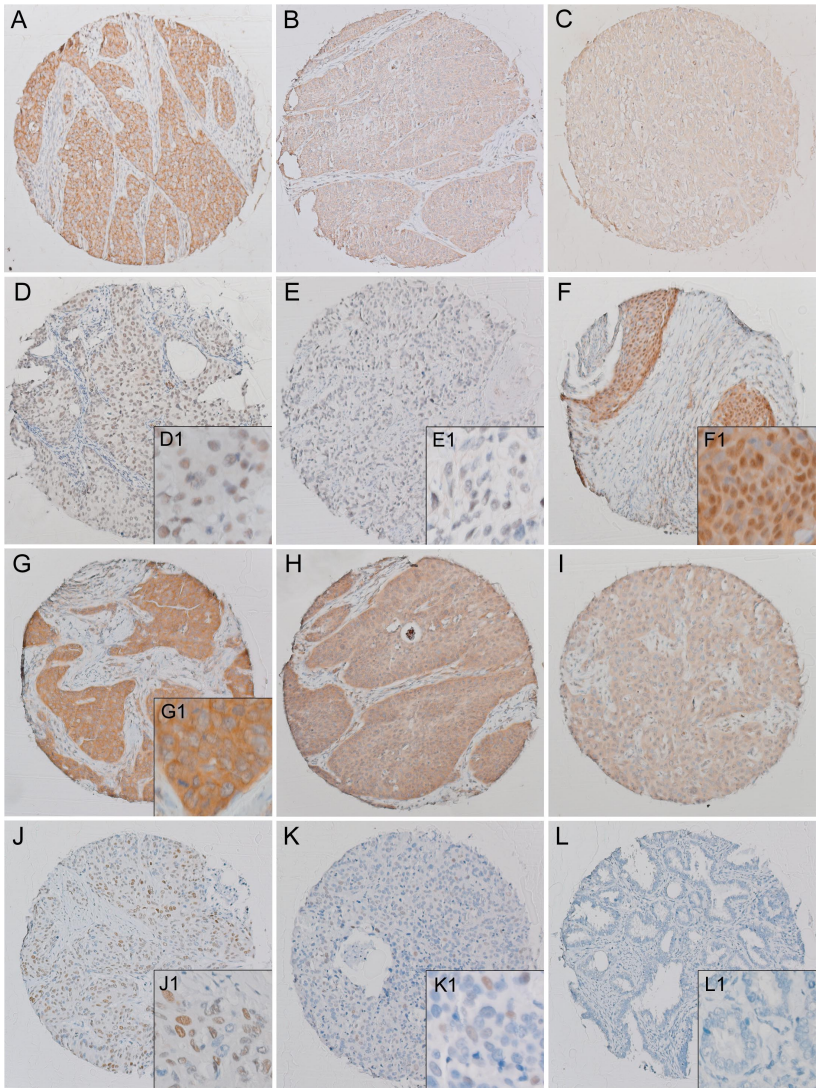


Chapter 3, Figure 2. LOH at 6p21.3. A) Microsatellite analysis at 6p of 63 samples, as described in Materials and methods. B) The percentage of LOH for each marker. C) LOH profile of tumor 31. This tumor contained a diploid and aneuploid subpopulation. The first panel represents the normal (V+K-) subpopulation of the tumor that is heterozygous for the three markers: D6S265, TY2A and TNF. The arrows point to the different alleles. The black peak, visible in the graphs of TNF, is a size marker. The second panel, represents the diploid tumor (V-K+) subpopulation with an allelic imbalance (A.I.) of ~2 at 6p, depicting an apparent decrease in one of the alleles for the 3 markers. The third panel shows complete loss of the allele for the three markers in the aneuploid tumor cells. The two graphs below illustrate the flow sorted subpopulations of the multiploid tumor. In green, the V+K- fraction, consisting of normal cells, is depicted (left graph). In red, the K+V- fraction, representing the tumor cells, is visible. As shown in the right graph, most of the tumor cells are aneuploid and a few are diploid, whereas the normal cells are all diploid.

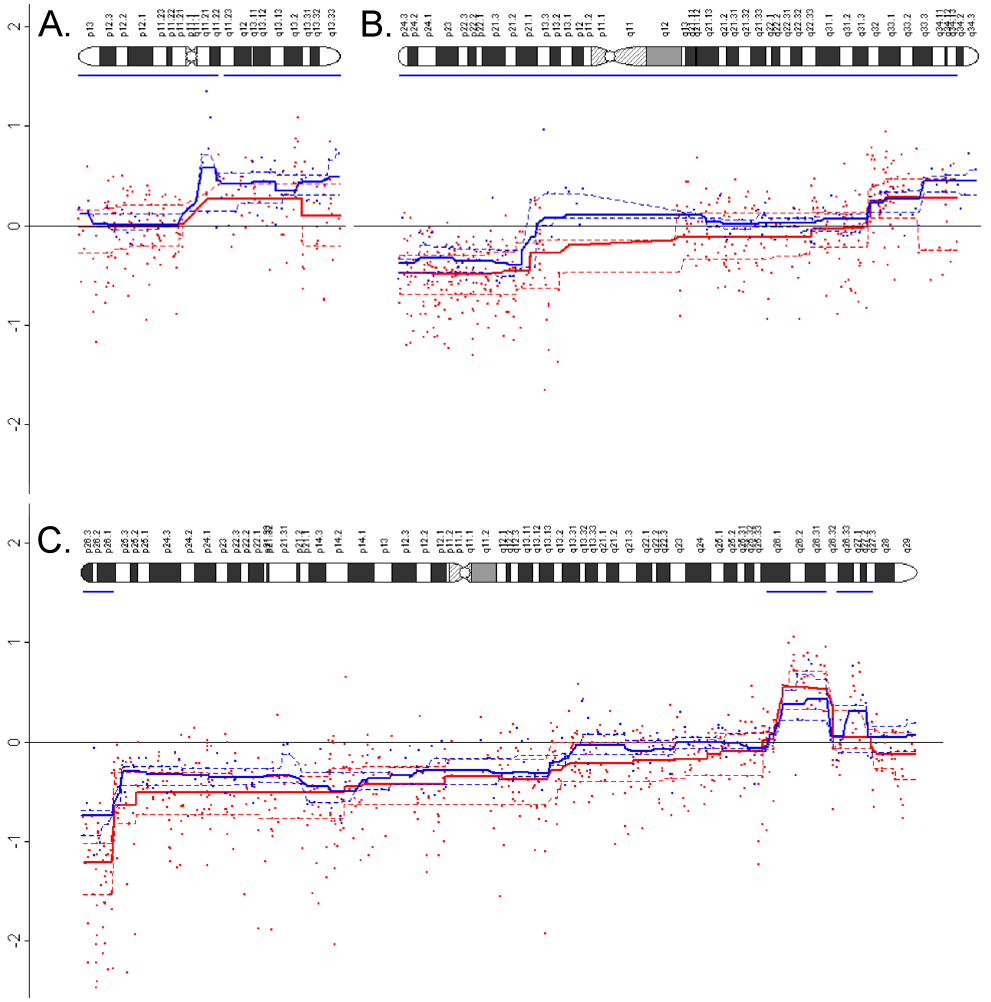
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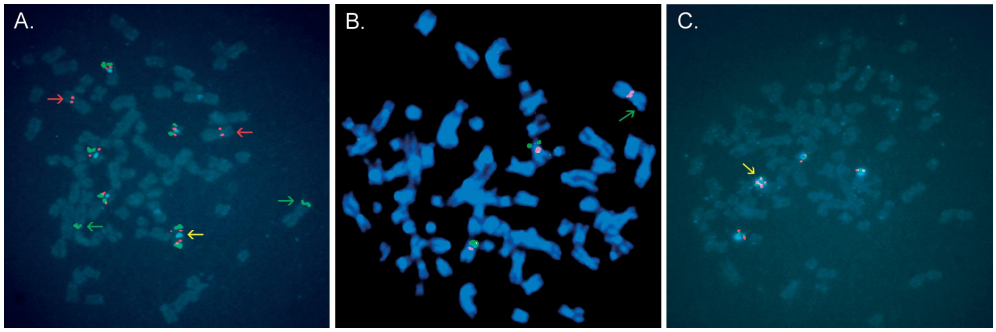
Chapter 4, Figure 4B. TGF-β1 induced changes in gene expression of pathways in HeLa, SiHa and CC10B. Pathways, shown in light gray boxes, are associated with TGF-β1 signaling and involved in regulation of cell proliferation. The boxes below the pathways show in red, genes which are upregulated in expression, in blue, genes which are downregulated and in black, genes which show evident upregulation and downregulation during the 4 time points compared to time point 0 hr. In HeLa SSCG are observed in MAPK, Smad, Wnt, TNFα, NFKB and Rho pathways, in SiHa SSCG are observed in MAPK, Smad, TNFα and NFKB pathways and in CC10B, SSCG were found in MAPK, Smad, TNFα, IFN, FGF and Rho pathways.



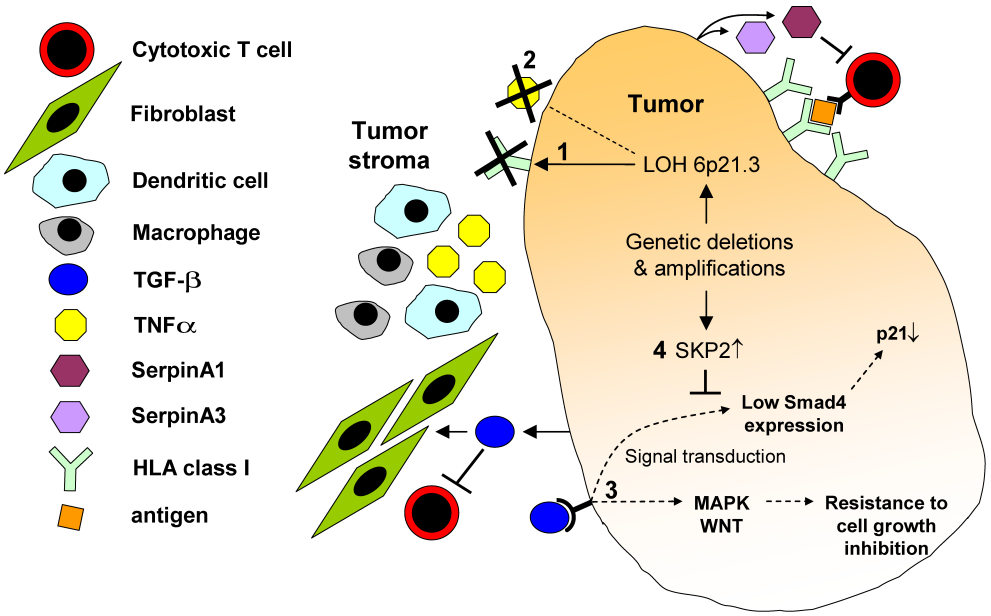
Chapter 5, Figure 1. Expression of Smad2, Smad2-P, Smad4 and p21 proteins in primary cervical carcinomas. A tissue-microarray of 117 cervical tumors was stained using immunohistochemistry as described in Materials and Methods (125x magnification). A: Smad2, strong cytoplasmic staining, B: Smad2, moderate cytoplasmic staining, C: Smad2, weak cytoplasmic staining, D: positive nuclear staining of Smad2-P in > 50% of tumor cells, D1: detail of D (400x magnification), E: Smad2-P nuclear staining in < 50% of tumor cells, E1: detail of E (400x magnification), F: Smad4, positive nuclear staining in > 50% of cells, F1: detail of F (400x magnification), G: Smad4, strong cytoplasmic intensity and absent nuclear staining, G1: detail of G (400x magnification), H: Smad4, moderate cytoplasmic intensity, I: Smad4, weak cytoplasmic intensity, J: p21 nuclear staining in > 50% of tumor cells, J1: detail of J (400x magnification), K: p21 nuclear staining in > 0% and < 50% of tumor cells, K1: detail of K (400x magnification), L: absent p21 nuclear staining and L1: detail of L (400x magnification).



Chapter 6, Figure 2. Complex genomic alterations of chromosome 20 (A) and chromosome 8 (B) of SiHa and chromosome 3 of CaSki (C). Below each chromosome ideogram, SNP array data are shown in blue and array CGH data in red. The blue line directly beneath the ideogram depicts LOH, whereas the blue line shifting around the baseline (0) shows copy number retrieved from SNP array data. The dotted blue and red lines represent the confidence intervals for the copy number of the SNP and the CGH array, respectively.



Chapter 6, Figure 3. FISH of metaphase preparations depicting copy number alterations. A) CaSki - 5 chromosomes 5 (centromere 5-blue; TRIO-green; SKP2-red); one isochromosome 5p (yellow arrow), 2 translocations of RP11-1150G22, encompassing TRIO (green arrow) and 2 translocations of RP11-624K2, encompassing SKP2 (red arrow). B) CaSki - 3 chromosomes 8 (centromere 8-red, TUSC3-green) with loss of one copy the BACs encompassing TUSC3, RP11-44L18/184C1 (green arrow). C) HeLa - 4 chromosomes 20 (centromere 20-blue, ZNF-217-red, CYP24A1-green); one isochromosome 20q (yellow arrow).



Chapter 7, Figure 1. Investigated immune escape mechanisms. 1. Cervical tumors show frequent loss of HLA class I expression. *Immune escape routes in HLA class I positive tumors* were investigated. HLA class I positive tumors showed strong expression of SerpinA1/SerpinA3 as a possible strategy of the tumor to escape immune surveillance (chapter 2). A common genetic alteration resulting in loss of HLA class I is loss of heterozygosity (LOH) at 6p21.3, the locus of HLA class I and TNFα. 2. *Loss of TNFα expression* due to LOH, mutation and/or promoter methylation was investigated. Genetic inactivation as a cause for lack of TNFα expression is unlikely (chapter 3). 3. *Loss of TGF-β sensitivity*. Resistance to TGF-β-induced growth inhibition is accompanied with stimulation of growth promoting pathways (MAPK and WNT) that could possibly counteract or overrule the growth inhibiting signal of TGF-β via Smads (chapter 4). Low cytoplasmic and absent nuclear staining of Smad4, a member of the canonical TGF-β pathway, may influence cervical carcinogenesis since it correlated with poor outcome (chapter 5), emphasizing the importance of the integrity of the canonical TGF-β pathway. 4. Performing genome-wide analysis (chapter 6), we observed *amplification and overexpression of SKP2*, a candidate oncogene which plays a role in TGF-β signaling since it needs to be degraded in order to accomplish TGF-β mediated cell cycle arrest.